Design and Synthesis of Pyrimidine Based Fused Heterocyclics in the Potential Treatment of Cancer and Opportunistic Infection

Farhana Islam

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DESIGN AND SYNTHESIS OF PYRIMIDINE BASED FUSED HETEROCYCLICS IN THE
POTENTIAL TREATMENT OF CANCER AND OPPORTUNISTIC INFECTION

A Dissertation
Submitted to the Graduate School of Pharmaceutical Sciences

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the degree of Doctor of Philosophy

By
Farhana Islam

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DESIGN AND SYNTHESIS OF PYRIMIDINE BASED FUSED HETEROCYCLICS IN THE
POTENTIAL TREATMENT OF CANCER AND OPPORTUNISTIC INFECTION

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ABSTRACT

DESIGN AND SYNTHESIS OF PYRIMIDINE BASED FUSED HETEROCYCLICS IN THE POTENTIAL TREATMENT OF CANCER AND OPPORTUNISTIC INFECTION

By

Farhana Islam

August 2020

Dissertation supervised by Dr. Aleem Gangjee

This dissertation designates an introduction, background and research progress in the areas of agents designed as (a) inhibitors of microtubule polymerization and multiple receptor tyrosine kinase (RTK) for potential treatment of cancer; (b) selective Pneumocystis jirovecii dihydrofolate reductase (pjDHFR) inhibitors for pneumocystis pneumonia (PCP) infection and (c) substrates for tumor-targeted therapy for cancer.

Dose-limiting toxicities of clinically used agents and development of resistance are two significant limitations in cancer chemotherapy. Microtubule targeting agents (MTAs) are a structurally diverse set of compounds that disrupt microtubule dynamics and exert their anticancer effect. Combination chemotherapy with antiangiogenic agents and microtubule targeting agents has shown an advantage against both these drawbacks. Single agents with dual antiangiogenic activity and cytotoxicity would afford a therapy that circumvents pharmacokinetic problems of
multiple agents, avoid drug-drug interactions, lower the drug dose, decrease overlapping toxicities, and delay or prevent tumor cell resistance. The work in this dissertation reflects the progress of fused pyrimidines, aimed to inhibit tubulin polymerization as well as act as antiangiogenic agents which inhibit one or more of the receptor tyrosine kinases (RTKs)- vascular endothelial growth factor receptor-2 (VEGFR2), platelet derived growth factor receptor-β (PDGFRβ) and epidermal growth factor receptor (EGFR), using molecular modeling studies. This work also reviews the synthesis of bicyclic and tricyclic thieno[2,3-d]pyrimidines and pyrazolo[4,3-d]pyrimidines and discusses novel synthetic strategies for substituted thieno[2,3-d]pyrimidines and pyrazolo[4,3-d]pyrimidines.

Pneumocystis pneumonia (PCP) is a form of pneumonia that is caused by the yeast-like fungus *Pneumocystis jirovecii*. The current treatment involving the use of a combination of trimethoprim and sulfamethoxazole is limited by drug resistance, treatment failures and adverse side effects. Newer drugs are critically needed for patients, unresponsive or resistant to this treatment. Dihydrofolate reductase (DHFR) is an essential enzyme that provides folate cofactor for DNA, RNA, and methionine biosynthesis. Hence, selectively inhibiting pjDHFR is an important strategy for effective treatment of infection by the pathogen. Structure-based design, using pjDHFR homology model and through the identification of amino acid differences between pjDHFR and hDHFR active sites, has been presented in the text. Novel synthetic strategies were developed for efficient synthesis of 6-(arylthio)pyrido[2,3-d]pyrimidine-2,4-diamines.

Three specialized folate transport systems exist in mammalian cells that include the reduced folate carrier (RFC), folate receptors (FRs) α and β, and the proton-coupled folate transporter (PCFT). Among several targeting strategies for cancer cells, selectively targeting through PCFT and FRs, over RFC have been successfully investigated. To avoid dose-limiting
toxicities, the next valid step in the field is to carry out a structure-based drug design to gain selectivity for PCFT and/or FRs transport over RFC. Drug discoveries in these areas become challenging in the absence of X-ray crystal structures for PCFT and RFC. PMX, the most widely used antifolate, has three disadvantages: (i) transport by RFC; (ii) dependence on its polyglutamylation for potency; and (iii) development of resistance due to mutagenesis in the target enzyme (thymidylate synthase). This dissertation focuses on the development of substituted-pyrazolo[4,3-\textit{d}]pyrimidines and pyrrolo[2,3-\textit{d}]pyrimidines to combat the above-mentioned drawbacks of PMX, using the X-ray crystal structures of intracellular targets and transporters and using the basic principles of scaffold hopping and bioisosteric replacements. The work described herein discusses our efforts to obtain agents with inhibition of two or more intracellular targets to inhibit \textit{de novo} purine biosynthesis. Synthetic efforts for the development of pyrazolo[4,3-\textit{d}]pyrimidines with different linkers have been discussed.
DEDICATION

Dedicated to my parents
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<tbody>
<tr>
<td>AIRS</td>
<td>5-amino-4-imidazole Ribonucleotide Synthase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-amino-4-imidazolecarboxamide ribonucleotide</td>
</tr>
<tr>
<td>AICARFTase</td>
<td>5-amino-4-imidazolecarboxamide ribonucleotide formyl transferase</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AK</td>
<td>Adenosine Kinase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>APRT</td>
<td>Adenine Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
</tr>
<tr>
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<td>Adenylosuccinate Lyase</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>CAIRS</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CBE</td>
<td>Chloride Bicarbonate Exchanger</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>coA</td>
<td>Co-enzyme A</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DHFR</td>
<td>Dihydrofolate Reductase</td>
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<tr>
<td>DHPS</td>
<td>Dihydropteroate Synthase</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N, N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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</table>
dTMP deoxythymidine monophosphate
dUMP deoxyuridine monophosphate
EGF Epidermal Growth Factor
FGAM Formyl Glycinamide Ribonucleotide Synthase
FGF Fibroblast Growth Factor
FPGS PolyPoly-γ-Glutamate Synthetase
FR Folate Receptor
GAR Glycinamide Ribonucleotide
GARFTase Glycinamide Ribonucleotide Formyl Transferase
GARS Glycinamide Ribonucleotide Synthase
GDP Guanosine Diphosphate
GGH γ-Glutamyl Hydrolase
GPAT Glutamine Phosphoribosylpyrophosphate Amidotransferase
GPI Glycosylphosphatidylinositol
GTP Guanosine Triphosphate
HGPRT Hypoxanthine-guanine Phosphoribosyl Transferase
HIV Human Immunodeficiency Virus
HPLC High-Performance Liquid Chromatography
HRMS High-Resolution Mass Spectrometry
IMP inosin-5′-monophosphate
LDA Lithium diisopropylamide
MCT Monocarboxylate-H⁺ Transporter
MDR Multidrug Resistant
<table>
<thead>
<tr>
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<tr>
<td>MFS</td>
<td>Major Facilitator Superfamily</td>
</tr>
<tr>
<td>MFT</td>
<td>Mitochondrial Folate Transporter</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MTHFD1</td>
<td>Methylene tetrahydrofolate dehydrogenase 1</td>
</tr>
<tr>
<td>MTHFD2</td>
<td>Methylene tetrahydrofolate dehydrogenase 2</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance Protein</td>
</tr>
<tr>
<td>MS</td>
<td>Methionine Synthase</td>
</tr>
<tr>
<td>MTA</td>
<td>Microtubule Targeting Agent</td>
</tr>
<tr>
<td>MTAP</td>
<td>Methylthioadenosine Phosphorylase</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene Tetrahydrofolate Reductase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target Of Rapamycin</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NBC</td>
<td>Na(^+)/HCO(_3)^(-) co-Transporter</td>
</tr>
<tr>
<td>NHE</td>
<td>Na(^+)/H(^+) Exchanger</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PAICS</td>
<td>Phosphoribosyl Aminoimidazole Succinocarboxamide Synthetase</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton-Coupled Folate Transporter</td>
</tr>
<tr>
<td>PCP</td>
<td>Pneumocystis Pneumonia</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>PIGF</td>
<td>Placental Derived Growth Factor</td>
</tr>
<tr>
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<td>Pralatrexate</td>
</tr>
<tr>
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<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMX</td>
<td>Pemetrexed</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PRPP</td>
<td>Phosphoribosyl Pyrophosphate</td>
</tr>
<tr>
<td>PTX</td>
<td>Piritrexim</td>
</tr>
<tr>
<td>RET</td>
<td>REarranged during Transfection</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced Folate Carrier</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SAICARS</td>
<td>5-aminoimidazole-4-(N-succinylocarboxamide) Ribonucleotide Synthase</td>
</tr>
<tr>
<td>SAM</td>
<td>S-Adenosyl-Methionine</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure Activity Relationship</td>
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<td>SHMT1</td>
<td>Serine Hydroxymethyl Transferase 1</td>
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<td>Serine Hydroxymethyl Transferase 2</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>SMX</td>
<td>Sulfamethoxazole</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-Associated Macrophage</td>
</tr>
<tr>
<td>TBAF</td>
<td>Tetra-$n$-butylammonium Fluoride</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
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</table>
TLC  Thin Layer Chromatography
TMD  Transmembrane Domain
TMP  Trimethoprim
TMQ  Trimetrexate
TS   Thymidylate Synthase
VDA  Vascular Disrupting Agent
VEGF Vascular Endothelial Growth Factor
I. BIOCHEMICAL REVIEW

A.1. Single agents with combination chemotherapy and multiple RTK inhibitory potential

A.1.1. Microtubules

A.1.1.1 Microtubule structure

Microtubules are crucial components of the cytoskeleton and are involved in critical cellular processes including cell division, trafficking, signaling and migration.\(^1\)

\[\text{Figure 1: Structure of microtubules: } \alpha\beta\text{-tubulin heterodimers assemble into protofilaments.}\]

\(^2\) Tubulin dimers polymerize to form microtubules and consist of 13 linear protofilaments assembled around a hollow core.\(^3\) Tubulin is globular protein and consists of five distinct families, the alpha (\(\alpha\)), beta (\(\beta\)), gamma (\(\gamma\)), delta (\(\delta\)), epsilon (\(\varepsilon\)) and zeta (\(\zeta\)). At a macroscopic level the
The diameter of the hollow tube is ~25 nm. The α-tubulin and β-tubulin are important members of the tubulin family that build the microtubule (Figure 1). These tubulin dimers associate longitudinally to form protofilaments. Lateral assembly of protofilaments forms the microtubule, which can be further elongated or shortened by single α- and β-tubulin heterodimers (Figure 1).

**Figure 2.** Structure, polymerization and depolymerization of microtubules.

At a critical concentration, where these dimers are present above the specific concentration, polymerization takes place and by the addition of dimer, this process is accelerated. However, microtubules possess a crucial feature of polarity, which is mandatory for its biological function. Therefore, in a protofilament, one end has the α subunits exposed. In contrast, the other end has the β subunits exposed, these ends designated as (α) and (β) ends, respectively (Figure 1). The β-tubulin subunit is exposed at the more dynamic plus-end of the microtubule, which typically extends toward the cell periphery. The microtubule minus-end is less dynamic and in cells, is commonly anchored at the centrosome-containing microtubule organizing center (MTOC).
adjacent to the nucleus which is exposed to the α tubulin. Both the α- and β-tubulin subunits bind GTP (Figure 2); however, only the nucleotide on β-tubulin is hydrolyzed and exchanged. At the site of (-) polarity, GTP molecule binds irreversibly and at the (+) end, GTP molecules bind reversibly.\textsuperscript{1,4} Microtubules undergo dynamic growth and shrinkage (Figure 2). During growth (polymerization), α and β monomer ends are bound by GTP. During shrinkage (depolymerization), the GTP bound at β tubulin end is hydrolyzed to GDP. The GDP-bound β tubulin at the tip causes disassembly of the microtubule. The switch from growth to shrinkage is called a catastrophe. On the contrary, the switch from shrinkage to growth is called rescue.\textsuperscript{4}

A.1.1.2 Microtubule Targeting Agents (MTAs)

Molecules binding to tubulin and interrupting tubulin dynamics are recognized as microtubule targeting agents (MTAs) and have been used clinically as single agents or in combinatorial regimens for the effective treatment of leukemia, lymphoma, and various solid tumors.\textsuperscript{3}

(a) Classification of MTAs:

Classification based on their effects on microtubule stability:\textsuperscript{5}

(i) Microtubule-destabilizing agents/ Microtubule depolymerizers: These microtubule destabilizers initiate microtubule depolymerization, leading to microtubule disassembly and a decrease in microtubule density. These include natural products such as the vinca alkaloids, cryptophycins, halichondrins, estramustine, colchicine and its analogs and combretastatines.
ii) **Microtubule-stabilizing agents/ Microtubule polymerizers:** These microtubule stabilizers promote the polymerization of tubulin, thereby increasing the density of microtubules. These include paclitaxel, docetaxel, epothilones and discodermolide.

**Figure 3.** Tubulin binding sites and representative agents of microtubule-targeted drugs.\(^3,4\)

**Classification based on binding domains of tubulin:**\(^3\)

Additionally, six groups of distinct classes of microtubule-binding agents are classified based on their binding domain (Figure 3).\(^3,4\) Two tubulin-binding sites have been identified as microtubule polymerizers, Taxane\(^7\) and Laulimalide site\(^8,9\). Four tubulin-binding sites have been identified for microtubule depolymerizers: the vinca site,\(^10\) the colchicine site,\(^11,12\) the maytansine site,\(^13\) and, more recently, the pironetin site.\(^14,15\) The binding sites are the following:
1) **Taxanes site agents:**

![Chemical structure of paclitaxel and derivatives](image)

Paclitaxel ($R=\text{PhCO}$) and docetaxel ($R=\text{BuOCC}$) are representative taxanes. Paclitaxel (Figure 4) was the first microtubule stabilizer identified. Paclitaxel and its derivatives and most MTAs that increase microtubule polymerization, such as the epothilones, discodermolide, eleutherobin, and sarcodictyins, bind to the Taxol-binding site. Ixabepilone, which belongs to the Epothilone class of paclitaxel-site binders, was approved for the treatment of drug-refractory metastatic breast cancer in 2007. All the agents bind to a pocket of β-tubulin located on the luminal side of microtubules (Figure 3). Paclitaxel is suggested to bind by diffusing through pores in the microtubule lattice. Binding of paclitaxel stabilizes the microtubule by...
inducing a conformational change in β-tubulin that increases its affinity for adjacent tubulin molecules.\textsuperscript{16}

2. Laulimalide/Peloruside agents:\textsuperscript{9}

![Laulimalide and Peloruside A](image)

**Figure 5.** Structure of Laulimalide and Peloruside A.

Laulimalide and peloruside A (Figure 5), are a sponge-derived natural product that induces tubulin hyper assembly.\textsuperscript{9} Laulimalide/peloruside site, are targeted by the named natural products. They have a unique binding site located on two adjacent β-tubulin units between tubulin protofilaments (Figure 3).\textsuperscript{19} The binding causes the bridging of two adjacent β-tubulin across the protofilaments.

3) The Vinca site agents:

![Representative structure of Vinca alkaloids](image)

**Figure 6.** Representative structure of Vinca alkaloids
Noble and Charles Thomas Beer were the first to identify vinca alkaloid from *Catharanthus roseus* (basionym Vinca rosea) family *Apocynaceae* in 1950. This class includes the Vinca alkaloids (Figure 6): vincristine, vinblastine, vinorelbine, vinflumine and vindesine. They have been widely used in cancer chemotherapy for leukemia, lymphomas and non-small cell lung cancer.\(^2\) The effect of such alkaloids can be concentration-dependent. At low concentrations, vinblastine inhibits microtubule assembly and depolymerization of microtubules, whereas at higher concentrations, vinblastine causes tubulin assembly. The Vinca alkaloids bind to the β-subunit of soluble tubulin heterodimers at a region commonly referred to as the Vinca-binding domain (Figure 3).\(^18,2^0\) Other MTAs such as the cryptophycins, halichondrins and dolastatins also bind at the same site.

4) Colchicine site agents\(^1^2\):

![Colchicine and combretastatin](image)

**Figure 7.** Colchicine and combretastatin

One of the most potent anti-mitotic agent which gained the attention of medicinal chemists and cancer biologists is combretastatin A-4 (CA-4, Figure 7).\(^2^1\) Colchicine binding site inhibitors have been extensively studied and many of them have entered clinical trials, including 2-methoxyestradiol, combretastatin A-4 phosphate (CA-4P, Figure 7) (fosbretabulin), the combretastain CA-1P prodrug (OXi4503), AVE8062 ((2S)-2-Amino-3-hydroxy-N-[2-methoxy-
5-[(Z)-2-(3,4,5-trimethoxyphenyl)ethenyl]phenyl]propenamide), CKD-516 ((S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide hydrochloride), BNC105P (sodium 6-methoxy-2-methyl-3-(3,4,5-trimethoxybenzoyl)benzofuran-7-yl phosphate), ABT-751 (N-[2-(4-hydroxyanilino)pyridin-3-yl]-4-methoxybenzenesulfonamide), CYT-997 (1-ethyl-3-[2-methoxy-4-[5-methyl-4-[(1S)-1-pyridin-3-ylbutyl]amino]pyrimidin-2-yl]phenyl]urea), ZD6126 (Phosphoric acid mono-(5-acetylamino-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-3-yl) ester), plinabulin (NPI-2358) ((3Z,6Z)-3-benzylidene-6-[(5-tert-butyl-1H-imidazol-4-yl)methylidene]piperazine-2,5-dione), MN-029 (methyl N-[6-4-[[2S)-2-aminopropanoyl]amino]phenyl]sulfanyl-1H-benzimidazol-2-yl]carbamate).

Colchicine binding site agents include podophyllotoxin, combretastatin and flavonols. Colchicine is not used clinically in cancer treatment due to its toxicity at doses that produce antimitotic effects. Colchicine binds to soluble tubulin at a distinct site referred to as the colchicine binding site. CA4P (Fosbretabulin) is under extensive investigation for platinum-resistant ovarian cancer, neuroendocrine tumors (NETs), hepatocellular carcinoma, and gastric cancer. The colchicine binding site is on β-tubulin at the interface between α- and β- tubulins (Figure 3).

5) Maytansine site agents:

The maytansine site (Figure 3) on tubulin has been discovered only very recently. It is a unique site on β-tubulin that is located at the longitudinal tubulin–tubulin interface in microtubules, which readily explains the microtubule-destabilizing effects of maytansine-site ligands. Three distinctly different ligands that target the maytansine site have been described: maytansine, PM060184 and rhizoxin (Figure 8).
Figure 8. Structure of Maytansine, Rhizoxin and PM-060184

Maytansine (Figure 8) has been successfully incorporated into the ADC trastuzumab emtansine, which is approved for the treatment of breast cancer.\(^{27}\) PM-060184 is in phase II clinical development for the treatment of breast cancers (clinicaltrials.gov), while rhizoxin had reached phase II, before being discontinued for reasons that are poorly understood.\(^{28}\)

6) Pironetin site agents:

Figure 9: Structure of Pironetin
Pironetin (Figure 9) is the only natural product crystallographically characterized to bind solely to α-tubulin. It was initially known as PA-48153C and was isolated concurrently from the fermentation broth and cell paste of *Streptomyces prunicolor*. Pironetin displays potent *in vitro* activity against cell lines both sensitive and resistant to current first-line therapeutics. Pironetin is cancer-cell specific, as it was inactive against normal human lung fibroblasts (IMR90) that displayed normal cell-cycle bias and no indications of apoptosis following treatment. Furthermore, pironetin is not a P-glycoprotein (Pgp) substrate.

A.1.1.3 Role and Functions of MTAs:

MTAs’ biological functions are due to their effects in polymerization dynamics.

A.1.1.3.1 Mitosis as a target:

Microtubules are ideal targets for anticancer drugs because of their pivotal role in mitosis, and have long been recognized as “anti-mitotic agents”. MTAs can either increase or decrease polymerization at different concentrations. The effect of mitotic arrest is due to an aberrant spindle formation. Both these effects cause cell-cycle arrest and cell death through apoptosis at the cellular level. Based on MTAs’ success as anti-mitotic agents, inhibitors targeting several mitotic kinases (Polo-like kinase, Aurora Kinase A and Aurora Kinase B) have been developed. Nevertheless, they did not show success in clinical trials. The hypothesis that MTAs target only mitosis has since been questioned and is supported by the findings that human tumors (sensitive to MTAs) *in vivo* do not divide as fast as *in vitro* tumor cells. As a result, other effects of MTAs in tumors are being investigated in addition to mitosis.
A.1.1.3.2 Interphase as a target:

Figure 10. Diverse anticancer interphase activities of MTAs.

Literature precedence suggests that MTAs act primarily on interphase cells. The microtubule is responsible for various functions at the interphase (Figure 10), namely: (a) centrosome clustering; (b) induction of voltage-dependent anion channel opening with release of Ca\(^{2+}\) and cytochrome c; (c) disruption of delivery of mRNA along interphase microtubule tracks; (d) induction of mRNA release from polysomes; (e) improvement of major histocompatibility complex (MHC) class I expression; increased activation of (f) dendritic cells, (g) cytotoxic T lymphocytes and (h) macrophages; (i) impede vesicular traffic to the cell front; (j) blocked timely endocytosis; (k) interfere with transcription factor transport by motors to the nucleus with (l) up- or down-regulation of tumor suppressor or oncogenes, respectively. The major dose-limiting toxicity, neurotoxicity, is because of the activity of MTAs on the interphase of the non-dividing neurons. However, the most important factors (Figure 10) responsible for cell death is yet to be
determined. Because there are a number of mechanisms by which MTAs operate, as illustrated in Figure 10, the chemotherapeutics work in the interphase are responsible for cancer cell death by prompting “thousand cuts” on the cancer cell. Knowledge of these targets and to identify the differences in interphases of normal and cancer cells are currently underway.

A.1.1.3.3 Vascular disruption:

The tumor vasculature is an attractive target for cancer therapy because their survival and the main route for metastatic spread require oxygen and nutrients by the vasculature. Tumor vessels are tortuous, strikingly heterogeneous and are an important feature of cancer. Multiple factors, including the specific organ and tissue in which the tumor arises, the composition of tumor-associated stromal cells, as well as the nature, diversity and relative abundance of pro- and anti-angiogenic mediators control the nature of tumors. Combretastatin analogs are recognized as vascular disrupting agents (VDAs). The tumor-VDA effects are seen well below the maximum tolerated dose of the combretastatins’. Tumor-VDA effects have advantages in solid tumors which have different vasculature of endothelial cells and pericytes than normal cells. Tumor cells are more permeable than normal cells because of the absence of vascular smooth muscle cells. Solid stress also compresses lymphatic vessels, further elevating interstitial fluid pressure and exacerbating the reduced blood flow caused by vessel leakiness. Endothelial cells rely on the tubulin cytoskeleton to maintain cell shape. Tumor-VDAs can cause rapid disruption of blood supply to solid tumors by disruption of vascular endothelial-cadherin at the junction. This changes the cell shape change, decreased blood flow to the tumor cells and vasoconstriction, causing tumor ischemia and cell death.
Figure 11. Differences between tumor-VDAs and antiangiogenic agents.\textsuperscript{49} (Modified from ref. 49)

The cytotoxic effect of vascular disruption is different from the cytostatic effect of antiangiogenic agents (Figure 11).\textsuperscript{49} VDAs present an alternative way to target tumor blood vessels. Unlike anti-angiogenic drugs, VDAs damage established tumor blood vessels.\textsuperscript{50} Tumor-VDAs cause tumor necrosis at the core of the tumor. Anti-angiogenic agents target neovascularization, prevent tumor growth and limit the tumors’ metastatic potential and thus their greatest effect is on the periphery of the tumor. Tumor-VDA effect and antiangiogenic effect have distinct profiles, offering potential for their use in combination.\textsuperscript{47} Compared to single agents, combination of tumor-VDA and antiangiogenic agent have produced delayed tumor growth in preclinical studies.\textsuperscript{47}

\textbf{A.1.1.4 MTAs Resistance Mechanism:}

The microtubule has been recognized as an effective target for the treatment of human malignancies.\textsuperscript{3} MTAs constitute a large group of chemically diverse compounds and are widely
used in the clinic for treatment of various types. However, despite the success, the clinical use of MTAs is often hampered by the acquisition of resistance. Cancer cells employ several distinct types of mechanisms to develop resistance to anti-microtubular drugs. Efflux via the membrane efflux pump of the ATP binding cassette (ABC) family is the primary mechanism of resistance towards MTAs. Pglycoprotein (Pgp), a product of a multidrug resistant gene (MDR1 or ABCB1) is responsible for the efflux of MTAs. The potency of paclitaxel was reduced 800-fold in Pgp-overexpressing SK-OV-3 MDR-1-6/6 cell line, compared to the parental cell lines. Pgp substrates cannot enter the CNS when drugs are administered orally. As a result, agents that are less susceptible to efflux by Pgp could possess a novel pharmacodynamic and pharmacokinetic profile. Microtubules are accumulated with more than 13 diverse isotypes of α and β tubulins. Among them, increased levels of βIII-tubulin are related with reduced rate of responses to taxanes in tumors of lung, breast and ovary. Contrary to the effect of βIII-tubulin with taxanes, epothilones are indifferent to the expression of βIII-tubulin.

A.1.2 Angiogenesis

Angiogenesis is the formation of new blood vessels, which involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels (Figure 12). Angiogenesis occurs during tumor development depending on changing tissue requirements. Blood vessels and stromal components respond to pro- and anti-angiogenic factors. However, in cancer, as the tumor grows, it obtains its blood supply. To achieve this, the tumor tilts the balance towards pro-angiogenic factors to drive vascular growth by attracting and activating cells from within the microenvironment of the tumor.
When a tumor grows beyond 2 mm³, it requires nutrients and oxygen for maintenance and growth. This phenomenon initiates the process of acquiring additional blood supply; and the process of angiogenic switching (from the release of anti-angiogenic factors to proangiogenic factors) in a tumor starts to develop (Figure 12). By releasing cytokines and growth factors, tumor cells exploit their microenvironment. These include vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDGF) and epidermal growth factor (EGF), which promote endothelial cell proliferation/migration. The new blood vessels grow and infiltrate the tumor to provide oxygen and nutrients to the growing tumor, leading to tumor progression and metastases. This process leads to a leaky tumor vasculature with irregular blood flow which makes it different from the normal tissue vasculature.

Figure 12. (a–e) Angiogenic “switch” from dormant cells to tumor vasculature.
Judah Folkman proposed the treatment of cancer by blocking angiogenesis nearly 45 years ago. Multiple anti-angiogenic agents has since been approved by the FDA as single agents or in combination with other cytotoxic drugs. Most notable of these are receptor tyrosine kinase inhibitors which are involved in the process of angiogenesis.

A.1.2.1 Receptor Tyrosine Kinase (RTKs):

Tyrosine kinases (TKs) play vital roles in diverse biological processes like growth, differentiation, metabolism and apoptosis in response to external and internal stimuli. TK can be divided into non-receptor TKs and receptor TKs (RTKs) where non-receptor TKs carry out intracellular communication and RTKs are involved in signal transduction. RTKs have three major parts: extracellular N-terminal domain, a transmembrane domain, and an intracellular C-terminal domain, which has the kinase activity. The kinase domain has a ATP-binding site between the N- and C-terminal lobes.

Figure 13. ATP binding site in RTK. (Modified from ref. 72-74)
Additionally, the ATP binding domain can be classified into the adenine region, sugar region and phosphate binding region (Figure 13). The C-terminal lobe forms an activation loop and has aspartic acid, phenylalanine and glycine, which is abbreviated as the “DFG” motif.

Dimerization (homodimerization or heterodimerization) and subsequent autophosphorylation of the tyrosine residues occurs upon binding growth factor on the extracellular domain of RTKs (Figure 14). The phosphorylations are tightly monitored and regulated under normal physiological conditions which is responsible for triggering a cascade of downstream cell signaling pathways. These pathways are highly dysregulated in cancer cells and inhibition of these can thus offer a key therapeutic strategy for the treatment of cancer.  

Figure 14. RTK signaling.
Figure 15. Representative small molecule RTK inhibitors

(a) **Epidermal growth factor receptor (EGFR):** \(^{78}\) There are eleven members and four EGFR family receptors in the EGF family. \(^{79}\) EGFR overexpression leads to an increased tumor cell proliferation, survival and invasiveness. \(^{80,79}\) EGFR signaling inhibition has been shown to promote selective apoptosis in tumor endothelial cells. \(^{81}\) Some examples of approved EGFR inhibitors are erlotinib (Tarceva®) (Figure 15) for locally advanced or metastatic non-small cell lung cancer, \(^{82}\) gefitinib (Iressa®) (Figure 15) for metastatic non-small cell lung cancer, \(^{83}\) and afatinib (Gilotrif®) (Figure 15) for squamous non-small cell lung cancer. \(^{84}\)

(b) **Vascular endothelial growth factor receptor (VEGFR):** \(^{85}\) The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) play significant roles in cancer. \(^{86}\) The
VEGFR family consists of three related RTKs- VEGFR-1, VEGFR-2 and VEGFR-3. The VEGF family has five members- VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental derived growth factor (P1GF). VEGF-A is the principal facilitator of the tumor angiogenesis and signals through VEGFR-2, which is the primary VEGF signaling receptor. VEGFR inhibitors are bevacizumab (Avastin®) for colorectal cancer, sunitinib (Sutent®) (Figure 15) for metastatic pancreatic neuroendocrine tumors and sorafenib (Nexavar®) (Figure 15) for advanced renal cell carcinoma.

(c) Platelet-derived growth factor receptors (PDGFR): The third most important factor involved in angiogenesis is the PDGFR. The PDGF has four members- PDGF-A, PDGF-B, PDGF-C and PDGF-D, which bind to receptors PDGFRα and PDGFRβ. The receptor PDGFRα signaling controls the development of several organs such as the lung, intestine, kidney, bones, and neuroprotective tissues. PDGFRβ signaling regulates hematopoiesis and blood vessel formation. PDGFs recruit pericytes and smooth muscle cells and promote the maturation and stability of the vasculature. Approved PDGFR inhibitor is imatinib (Gleevec®) (Figure 15) for chronic myeloid leukemia.

Resistance and severe toxicities are the two major problems associated with the use of RTK inhibitors as anti-angiogenic agents. Acquired and inherent resistance are frequently observed in preclinical and clinical settings. Bleeding, hypertension and fatigue are also common toxicities of RTK inhibitors. Resistance to anti-VEGF therapy, a non-VEGF could be responsible for angiogenesis signals in tumors. Thus, a combination approach targeting different angiogenesis signaling mechanisms could be highly effective. Additionally, synergism was observed in signaling by fibroblast growth factor-2 (FGF-2) and PDGF-BB in tumor growth and metastasis. Numerous small-molecules function as single agents with multi-RTK inhibitory potential. Axitinib
(Inlyta®) is approved for renal cell carcinoma which inhibits VEGFR, PDGFR and c-KIT. Vandetanib (Caprelsa®) is approved for the advanced medullary thyroid cancer which inhibits VEGFR2 and EGFR. Nindetanib (Ofev®) is approved for non-small cell lung cancer which inhibits VEGFR, FGRF and PDGFR. These clinically successful RTK inhibitors validate the development of single agents as multi-RTK inhibitors.

**A.1.3 Anti-angiogenic agents and MTAs as a combination chemotherapy**

Combination therapy, combines two or more therapeutic agents, is a mainstay in cancer therapy. Combination therapy could circumvent pharmacokinetic problems of multiple agents, avoid drug-drug interactions, and could be used at lower doses to minimize toxicities and tumor cell resistance than two or more agents dosed separately in combination chemotherapy. The combination therapy has several advantages: increased patient compliance, emergence of an additive or synergistic interaction of the combined drugs, prevention of resistance, reduction of drug dosage with a consequence of diminished toxicity. Inhibition of a single target or pathway is known to be of limited benefit to a cancer patient. Thus, combination therapy can provide an effective technique to combat resistance and target tumor heterogeneity.

**A.1.3.1 Vessel normalization for cancer**

MTAs, which are cytotoxic agents, kill cancer cells directly, whereas cytostatic agents like antiangiogenic agents kill cancer cells indirectly by the deprivation of nutrients and oxygen. A combination of cytotoxic drug with antiangiogenic agents showed clinical success in the treatment of cancer. Instead of blocking the delivery of cytotoxic drug to the tumor, the anti-angiogenic
therapy has shown to enhance its effect. Jain and coworkers\textsuperscript{102} established the “vascular normalization” hypothesis based on this observation.

![Diagram of changes in tumor vasculature during the course of anti-angiogenic therapy](image)

**Figure 16.** Schematic of changes in tumor vasculature during the course of anti-angiogenic therapy. \textit{a}, Normal vasculature, composed of mature vessels and maintained by the perfect balance of pro- and anti-angiogenic molecules, might not change during the course of anti-angiogenic therapy. \textit{b}, Abnormal tumor vasculature, composed largely of immature vessels with increased permeability, vessel diameter, vessel length, vessel density, tortuosity and interstitial fluid pressure, compromises the delivery of therapeutics and nutrients. \textit{c}, Judiciously applied direct or indirect antiangiogenic therapies might prune immature vessels, leading to more normalized tumor vasculature. This network should be more efficient for the delivery of therapeutics and nutrients. \textit{d}, Rapid pruning of, or coagulation in, tumor vasculature might reduce the vasculature to the point that it is inadequate to support tumor growth and might lead to tumor dormancy. This is the ultimate goal of anti-angiogenic/anti-vascular therapy.\textsuperscript{102}
Cancer cells have a hostile tumor microenvironment, disorganized blood vessels, which are leaky and tortuous (Figure 16). This conditions produce spatial heterogeneity in blood flow, low pH, increased interstitial fluid pressure and hypoxia. The blood flow heterogeneity decreases the delivery of cytotoxic drug. Acidic pH decreases activity of immune cells and hypoxia renders cells resistant to radiotherapy. An anti-angiogenic therapy can cause a transient increase in vascular patency, decrease in interstitial fluid pressure and hypoxia. The tumor angiogenic signal obstruction results in pruning of the immature blood vessels and remodeling of the vasculature. It thus normalizes the tumor blood vessels and improves blood flow, oxygen supply, and drug delivery to the tumor.

**Figure 17.** Proposed effect of drug dose and schedule on vascular normalization of tumor.

Under physiological conditions, the blood vessels have normal structure and function because of a balance of the signals downstream of the proangiogenic molecules (e.g., VEGF and Ang2) and antiangiogenic molecules (e.g., sVEGFR1, thrombospondins, and semaphorins). In contrast, under pathological conditions, tumor vessels are structurally and functionally abnormal because of an imbalance between pro- and antiangiogenic signals. This creates an abnormal
microenvironment in tumors, characterized by hypoxia, acidosis, and elevated fluid pressure, that fuels tumor progression and treatment resistance via multiple mechanisms as shown in Figure 17. Inhibiting proangiogenic signaling or enhancing antiangiogenic signaling can prune some abnormal vessels and remodel the rest, resulting in a normalized vasculature. Depending on the extent of normalization versus pruning, tumor perfusion/oxygenation may increase, remain unchanged, or decrease. Some tumors might be intrinsically resistant to a given AA agent, and others may switch to nonsprouting mechanisms of vessels recruitment that are refractory to the given AA agent and continue to make abnormal vessels again. (B) The window of increased perfusion from vascular normalization depends on the dose and potency of antiangiogenic therapy. High doses may cause excessive pruning of tumor vessels, resulting in a shorter window, and may starve a tumor of oxygen and other nutrients. High doses may also increase toxicity, including some fatal. The tumor vasculature normalization is limited to a fixed time after the antiangiogenic therapy is administered. This is defined as the “vascular normalization window” (Figure 17). This window is short-lived and delivery of cytotoxic drugs in this time duration can provide synergistic effect. Successful chemotherapy treatment requires lateral dosing, scheduling, sequencing and studying the tumor vasculature. To optimize the efficacy and reduce the negative side-effects of combination of cytotoxic drug and anti-angiogenic agent, careful consideration is mandatory.
A.1.3.2 Combination of vascular disrupting agents and anti-angiogenic agents

The VDAs target the existing tumor vasculature leading to central necrosis of the tumor. This leaves viable, proliferating cells at the periphery. These proliferating cells at the periphery can cause metastasis and thus limit the use of tumor-VDAs as monotherapy. As anti-angiogenic agents target the periphery, this phenomenon warrants the use of a combination therapy of VDAs with anti-angiogenic agents.

A.1.3.3 Multitargeted agents with MTA and RTK inhibitory potential

The multi-targeted drugs have much more potential and defined as rationally designed single chemical entities capable of selectively targeting two or more biological targets or processes. Multitargeted drugs have numerous advantages over combination chemotherapy, which includes (i) predictable pharmacodynamic and pharmacokinetic effects; (ii) improved patient compliance; and (iii) presence of multiple entities (in one agent) with multiple effects simultaneously. Combination chemotherapy entails a very careful selection of scheduling, which can be avoided during the design of a multitargeted drug. Drug delivery to the tumor can be improved via the surviving vasculature, while administrating a cytotoxic agent during the transient phase of tumor vasculature “normalization”. Dosing separate antiangiogenic and cytotoxic agents might miss the timing window of the transient normalization in combination chemotherapy. Single molecular entities with both antiangiogenic and cytotoxic features allow the cytotoxicity to be established as soon as the antiangiogenic effect and vasculature normalization occur.
In phase III clinical trial, Docetaxel (MTA) plus nintedanib (VEGFR, PDGFR and FGFR inhibitor) versus docetaxel plus placebo in patients in non-small-cell lung cancer (LUME-Lung 1) displayed an increase in overall survival of 10.9 months for the drug combination, compared to 7.9 months for docetaxel monotherapy. The clinical evidence prompted further investigation into the combination therapy of MTAs with multi-RTK inhibitors.

Figure 18. Reported 7-benzyl-N-(substituted)-pyrrolo[3,2-d]pyrimidin-4-amine as single agents acting as MTAs, along with inhibitors of VEGFR-2, PDGFRβ and EGFR. Pavana et al. reported 7-benzyl-N-(substituted)-pyrrolo[3,2-d]pyrimidin-4-amines 1-3 (Figure 18). These compounds act as a multitargeted single agents with MTAs and VEGFR-2, PDGFRβ and EGFR inhibition. Compound 2 displayed excellent inhibition across all the four targets among the three compounds. It has an IC\textsubscript{50} of 480 ± 8 nM in inhibiting tubulin assembly. The IC\textsubscript{50} against VEGFR-2, PDGFRβ and EGFR was 33 ± 5.0 nM, 2.3 ± 0.3 nM and 10.3 ± 1.7 nM respectively. Compound 2 also significantly reduced tumor growth in 4T1 cells (mouse mammary carcinoma), compared to paclitaxel.
Figure 19. Reported 5-methyl-furo[2,3-\(d\)]pyrimidines as single agents acting as MTAs, along with inhibitors of VEGFR-2, PDGFR\(\beta\) and EGFR.\(^{111}\)

Devambatla \textit{et al.} reported 5-methyl-furo[2,3-\(d\)]pyrimidines 4-6 (Figure 19) as multitargeted single agents acting as MTAs, along with inhibitors of VEGFR-2, PDGFR\(\beta\) and EGFR. Compounds 4-6 displayed nanomolar inhibition of EGFR kinase. Compounds 4-6 inhibited VEGFR-2 and PDGFR-\(\beta\) kinases better than or equal to the standard sunitinib. Gangjee and coworkers\(^{111, 112}\) have further reported 4-substituted-2,6-dimethylfuro[2,3-\(d\)]pyrimidines as multitargeted single agents that inhibit VEGFR-2, PDGFR\(\beta\) and EGFR, along with tubulin assembly. These studies attest to the rationale of designing compounds with anti-angiogenic and MTA potential to produce combination chemotherapy as single agents.

A.2. Selective pjDHFR inhibitors

A.2.1. \textit{Pneumocystis jirovecii} and pneumocystis pneumonia Infection

\textit{Pneumocystis pneumonia} (PCP) is a form of pneumonia that is caused by the yeast-like fungus \textit{Pneumocystis jirovecii} (pj).\(^{113}\) Pj is an opportunistic pathogen that causes severe pulmonary infection in immune compromised hosts.\(^{114}\) PCP commonly occurs in patients with human
immunodeficiency virus (HIV) infection. PCP can occur in patients without HIV, including those who have undergone organ transplantation, chemotherapy, and/or high-dose corticosteroid therapy.\(^\text{115}\) If left untreated, PCP is almost always fatal. Even when treated, the mortality rate is 10%. In the USA, 9% of HIV infected patients and 1% of organ transplant recipients develop PCP infection.\(^\text{116,117,118}\) It is an atypical fungus, which differs from other fungi in the fungi family due to the presence of cholesterol, instead of ergosterol, in its cell membrane. The species affecting humans is unique than the species affecting other animals such as rats and mice and the infection is host-specific. The infection from rats, when transfected to mice, does not proliferate, whereas the infection from rats to other rats causes severe infection.\(^\text{119}\) The infection remains under control in the healthy immune system of an individual. PCP presents itself when the patients’ CD4 count is below 200 cells/mm.\(^\text{120,121}\) Although PCP prophylaxis and antiretroviral therapy (ART) has changed the face of the HIV/AIDS epidemic, the incidences of HIV cases persist due to nonadherence to the medication, toxicity to the medications, emergence of drug-resistant HIV strains, late diagnosis of HIV and the rise of the number of cases in developing countries.\(^\text{122,123}\) The mortality rates of PCP range from 10% to 20% among HIV infected patients, while it is 30%–60% among the non-HIV population. Thus, PCP continues to be a significant public health concern. In the US, 9% of hospitalized HIV/AIDS and 1% of organ transplant patients develop PCP infection.\(^\text{124}\) In these patients, the mortality rate is from 5-40% while being treated for PCP and approaches 100% if left untreated.\(^\text{124}\)

For past few decades, the first-line therapy for both the prophylaxis and the treatment for PCP is Sulfamethoxazole/trimethoprim (SMX/TMP) combination (co-trimoxazole) (Figure 20).\(^\text{125}\) This combination synergistically blocks folic acid synthesis, since TMP is a selective, but weak inhibitor of dihydrofolate reductase (DHFR),\(^\text{126}\) while SMX is an inhibitor of dihydropteroate
synthase (DHPS).\textsuperscript{127} The low activity of TMP against DHFR is amplified by SMX, in the treatment regimen. The efficacy, low cost and activity in variety of infections has drove cotrimoxazole to be used indiscriminately. Due to the widespread use, there are mutations in the DHPS locus of \textit{pj} encoding DHPS as the cause of TMP/SMX resistant strains of PCP.\textsuperscript{127-130}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure20.png}
\caption{Clinical and non-clinical agents for the treatment for PCP.}
\end{figure}

Clinical and non-clinical mutations have also been reported in DHFR after treatment or prophylaxis using DHFR inhibitors.\textsuperscript{131-135} Due to such resistant strains or toxicity/allergy by SMX (Figure 20), treatment failure and discontinuation of co-trimoxazole occurs in several cases have been reported.\textsuperscript{136-140} Treatment failure to TMP/SMX has enabled TMP-dapsone or clindamycin-primaquine as the second-line treatment in mild to moderate PCP. This therapy leads to low efficacy and often lethal side-effects.\textsuperscript{141-144} Piritrexim (PTX, Figure 20) and trimetrexate (TMQ, Figure 20) are potent, but non-selective inhibitors of DHFR, which cause dose-limiting
toxicities.\textsuperscript{141, 145, 146} These agents, combined with leucovorin had high costs, drug toxicities, drug interactions and lack of efficacy.\textsuperscript{147} New drugs for the treatment of PCP are critically needed for patients that do not respond to first line treatment as well the inevitable recurrence of resistance.

**A.2.2. DHFR**

![DHFR Reaction](image)

**Figure 21. Reaction catalyzed by DHFR\textsuperscript{31}**

DHFR is an enzyme catalyzes the reduction of dihydrofolate to tetrahydrofolate (THF), using NADPH as an electron donor, and can be converted to the kinds of tetrahydrofolate cofactors used in 1-carbon transfer (C1 metabolism) chemistry (Figure 21).\textsuperscript{148, 149} DHFR is considered as an important drug target for several human diseases- protozoal, bacterial, fungal infections, and autoimmune diseases.\textsuperscript{150} Folate metabolism (C1 metabolism) has been studied for decades as a target in chemotherapy.\textsuperscript{151} Thymidine is synthesized by eukaryotic organisms via the thymidylate cycle, which consists of enzymes: (a) Serine hydroxymethyltransferase (SHMT); (b) DHFR; and (c) Thymidylate synthase (TS). DHFR catalyzes the NADPH-dependent reduction of 7,8-
dihydrofolate to the 5,6,7,8-tetrahydrofolate (THF) (Figure 21). The THF then acts via 5,10-methylene THF as a cofactor in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), catalyzed by TS. Inhibition of TS and/or DHFR causes disruption in DNA, RNA and protein synthesis of the organism and eventually leads to death. As a result, TS or DHFR inhibition causes “thymineless death” and which is a proven concept and drug discovery methodology for several antimalarial, antiprotozoal and antimicrobial agents.11

A.2.3. hDHFR, pcDHFR and pjDHFR

Pneumocystis is an unusual host-specific fungus that takes advantage of a weakened immune system.152 Pneumocystis carinii DHFR (pcDHFR) was presumed to be the causative species of PCP infection in humans and thus far, most of the drugs synthesized and evaluated for PCP infections were tested against pcDHFR.126, 153 On the other hand, pc however is a distinct species that infects rats, different from pj, responsible for human infections.134 There is a 38% amino acid sequence difference in the pcDHFR and pjDHFR.113 Therefore, drugs assessed and studied against the surrogate pcDHFR in-vitro, may not translate into activity in the treatment of PCP infection in humans.154 Additionally, due to a lack of crystal structure information for pjDHFR the rational design of pjDHFR inhibitors is hindered. The inability to grow the pj organism outside the human lungs impedes the drug discovery process. Therefore, there is an unmet need to develop a tissue culture for in vitro studies or an animal model for in vivo evaluation of the synthesized compounds for pjDHFR enzyme.
Table 1. Recombinant DHFR from pjDHFR and human DHFRs’ (hDHFR) inhibitory Concentrations (IC50, in nM) against and Selectivity Ratios154

<table>
<thead>
<tr>
<th></th>
<th>pjDHFR (IC50, in nM)</th>
<th>hDHFR (IC50, in nM)</th>
<th>h/pj</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2.4</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>5.6</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>4.2</td>
<td>150</td>
<td>35</td>
</tr>
<tr>
<td>TMP</td>
<td>120</td>
<td>32200</td>
<td>268</td>
</tr>
<tr>
<td>PTX</td>
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<tr>
<td>TMQ</td>
<td>2.1</td>
<td>2.6</td>
<td>1</td>
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</table>

These assays were carried out at 37 °C under 18 μM dihydrofolic acid concentration. The standard error of the means for these values is 12% or less than the mean value.

Gangjee and coworkers154 reported pyrido[2,3-d]pyrimidines 7-9 as selective and potent pjDHFR inhibitors (Table 1). Cody et al.155 reported the X-ray co-crystal structures of 9 with pcDHFR and hDHFR (PDB: 4IXE and 4QJC, respectively) to exemplify the interactions that defines the enhanced selectivity and potency. The crystal structures of 7-9 were reported as ternary complexes with either hDHFR or native pcDHFR.155 The hDHFR-9-NADPH ternary complex superposed with of pcDHFR-9-NADPH ternary complex crystal structure (Figure 22) discloses the differences and the binding geometry of 9 in pcDHFR and hDHFR.155 In hDHFR cocrystal structure (PDB:4QJC) with ligand 9 (cyan), the scaffold pyrido[2,3-d]pyrimidine is stabilized by a π-π stacking interaction with Phe31. The protonated N1 and 2-NH2 form an ionic bond with Glu30. The 4-NH2 forms hydrogen bond interaction between the backbone carbonyls of Ile7 and Val115. The side chain trifluorophenyl ring is
oriented in the pocket formed by Asp21, Ser59, Pro61 and Asn64. The $N^6$-CH$_3$ group is positioned towards Val115 side chain.

**Figure 22.** Superimposition of X-ray co-crystal structures of active sites of hDHFR (PDB: 4QJC, 1.62 Å) and pcDHFR (PDB: 4IXE, 1.54 Å). The hDHFR amino acid residues are displayed in pink, ligand $N^6$-methyl-$N^6$-(3,4,5-trifluorophenyl)pyrido[2,3-d]pyrimidine-2,4,6-triamine 9 is displayed in cyan and the NADPH in yellow. The pcDHFR amino acid residues are displayed in grey, ligand $N^6$-methyl-$N^6$-(3,4,5-trifluorophenyl)pyrido[2,3-d]pyrimidine-2,4,6-triamine 9 is displayed in magenta and the NADPH in tan color. The visualization was in Schrödinger Maestro 2020-1.

In the pcDHFR co-crystal structure (PDB: 4IXE) with ligand 9 (magenta) the protonated N1 and 2-NH$_2$ form an ionic bond with Glu32. The 4-NH$_2$ forms hydrogen bonding interaction between the backbone carbonyls of Ile10 and Ile123. The side chain trifluorophenyl ring is placed in the pocket formed by Ser24, Ser64, Pro66 and Phe69. The
\(N^\theta\text{-CH}_3\) group is oriented towards the Ile123 side chain. The amino acids that are different between the active sites of pcDHFR and hDHFR are indicated in Figure 22.

Figure 23. Sequence alignment of hDHFR (186 residues), pcDHFR and pjDHFR (both 206 residues). Mutational sites are underlined and in red while key active site residues are numbered and colored cyan Reproduced with permission of the International Union of Crystallography from ref.\textsuperscript{155} Copyright 2015 International Union of Crystallography.

Cody \textit{et al.}\textsuperscript{135} investigated the variability in pcDHFR, pjDHFR and hDHFR enzyme sequences. Residues seem to impact the active site (as seen in Figure 23) are described. In the hDHFR enzyme, these residues are Gln and Asn; in pcDHFR enzyme, these are Lys and Phe and in pjDHFR enzyme are Ser and Ser. Structural data for inhibitor binding to hDHFR and pcDHFR
reveal that the residues interact with inhibitors and that inhibitors have been designed in the past to specifically target these residues.134, 149, 157

**Table 2.** Kinetic constants (Ki) of TMP against pjDHFR, pcDHFR, and wild type and mutant hDHFR.135

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ki (nM)</th>
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<tbody>
<tr>
<td>hDHFR</td>
<td>5200 ± 700 (n = 4)</td>
</tr>
<tr>
<td>pcDHFR</td>
<td>800 ± 300 (n = 7)</td>
</tr>
<tr>
<td>pjDHFR</td>
<td>43 ± 5 (n = 14)</td>
</tr>
<tr>
<td>hDHFR Q35S</td>
<td>1800 ± 100 (n = 3)</td>
</tr>
<tr>
<td>hDHFR N64S</td>
<td>170 ± 0.5 (n = 2)</td>
</tr>
<tr>
<td>hDHFR Q35S/N64S</td>
<td>1200 ± 100 (n = 4)</td>
</tr>
</tbody>
</table>

TMP is safe in PCP infection and is comparatively inactive against hDHFR (Table 2) which is used as a potent pcDHFR and pjDHFR inhibitor.158 The importance of the positions in hDHFR was illustrated by evaluating the Ki of TMP against wild type and mutants of hDHFR (Table 2). The Q35S mutant hDHFR shows a 3-fold reduction in Ki for TMP, compared to hDHFR. The N64S mutant displays a 31-fold reduction in Ki compared to hDHFR. The Q35S/N64S double mutant displays a 4-fold reduction in Ki compared to hDHFR. Thus, pjDHFR over hDHFR selectivity can be achieved by rational design of compounds targeting the different amino acid residues in hDHFR and pjDHFR enzymes.
A. 3. Multitargeting single agents that target tumors via cellular uptake by Folate receptors and/or Proton-Coupled Folate Transporter and inhibit *de novo* purine nucleotide biosynthesis

### A.3.1 Folates

Figure 24. Physiologically important folates

Folate belongs to water-soluble B-vitamin and enzymatic cofactor that is necessary for the synthesis of purine and thymidine nucleotides.\(^\text{159}\) Folic acid can be described as the parent structure of folates (Figure 24). The group encompasses various oxidized forms of folic acid with substitutions at \(N^5\) and/or \(N^{10}\), and the presence of \(\gamma\)-linked glutamyl residues (2-8). Reduced folates (e.g. 5-methyltetrahydrofolate) are cofactors in the biosynthesis of purines and pyrimidines. Mammalian cells do not have the enzymatic machinery to create folates *de novo*, thus, folates are gained from dietary sources in mammals.\(^\text{160}\) Folates are hydrophilic and anionic structures that
cannot cross biological membranes by passive diffusion. As a result, for the absorption of folic acid several sophisticated transport systems are required.\textsuperscript{160}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure25.png}
\caption{Physiological role of folates in the cytosol.\textsuperscript{161}}
\end{figure}

\subsection*{A.3.2 Physiological roles of Folates}

Folates are essential cofactors and play a key role in cellular one-carbon C1 transfer reactions (Figure 25).\textsuperscript{162, 163} The reduced cofactor 10-formyl-THF is used in the purine biosynthesis in the cytosol.\textsuperscript{162, 164} The enzyme glycinamide ribonucleotide formyltransferase (GARFTase) utilizes 10-formyl-THF for the development of the imidazole ring of purines.\textsuperscript{164} 5-Aminomidazole-4-carboxamide ribonucleotide formyltransferase (AICARFTase) enzyme, uses 10-formyl-THF to synthesize inosin-5’-monophosphate (IMP).\textsuperscript{164} On the other hand, 5,10-methylene THF acts as a co-factor in reactions catalyzed by thymidylate synthase
The conversion of 2′-deoxyuridine monophosphate (dUMP) to 2′-deoxythymidine monophosphate (dTMP) is catalyzed by TS and the reaction generates DHF. The DHF is recycled into THF by the enzyme dihydrofolate reductase (DHFR), which is profusely expressed in the cytosol. Another form of folic acid, 5-CH₃-THF synthesized by Methylene Tetrahydrofolate Reductase (MTHFR) from 5,10-methyleneTHF in the cytosol acts as a cofactor for the reaction catalyzed by Methionine Synthase (MS). MS causes the conversion of cysteine to methionine, which later conjugates with Adenosine Triphosphate (ATP) to form S-Adenosyl-Methionine (SAM). SAM aids as a methyl group donor for several methylation reactions on DNA, RNA, histones and neurotransmitters, and thus can cause an increase or decrease in transcription and translation of proteins, such as tumor suppressor genes or promoter genes.

![Diagram](image.png)

**Figure 26.** The physiological role of the mitochondrial folates.

Transport of THF cofactor into mitochondria from the cytosol occurs via mitochondrial folate transporter (MFT/SLC25A32). THF is used mainly in the biosynthesis of formate and glycine in the mitochondria (Figure 26). For various cellular synthetic reactions the intermediate
folic acid derivatives are transported to the cytosol.\textsuperscript{170} Around 40% of the folate ares accumulated by mitochondria in the cell.\textsuperscript{170,171} DNA synthesis, DNA repair, DNA methylation, can be affected by abnormalities in the nucleotide biosynthesis, methylation reactions, glycine biosynthesis. This can cause genomic instability of the cellular system, potentially leading to cell death.\textsuperscript{172} The reactions 1, 3, 4, and 5 in both the mitochondria and cytoplasm are illustrated in Figure 26. Reaction 2 is limited only to the mitochondria. The enzymes involved are Serine hydroxymethyltransferase (SHMT) (1); glycine oxidase complex (2); 5,10-methylenetetrahydrofolate dehydrogenase (MTHF) (3); methenyltetrahydrofolate cyclohydrolase (MDMC ) (4); and formyltetrahydrofolate synthetase (FTHFS) (5).

\textbf{A.3.3 Transport of cellular folate}

\textbf{A.3.3.1 Transporters for folate influx}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Intracellular and extracellular transport of (anti)folates.\textsuperscript{162}}
\end{figure}
(a) Reduced Folate Carrier (RFC, SLC19A1): 162

RFC (Figure 27) is a group of transport proteins that is part of the major facilitator superfamily (MFS) of the transporter, specifically solute carrier (SLC) family of facilitative carriers. 173 The protein has 12 hydrophobic transmembrane domains (TMD) with a short hydrophilic N-terminus and long hydrophilic C-terminus in the cytosol. 174 RFC is a 591- amino acid transmembrane protein with a molecular mass of approximately 85 kDa. 174 There is no ATP binding site domain in RFC and thus, its transport is not ATP-driven. 174 It is a bidirectional antiporter that exchanges adenosine nucleotides, thiamine monophosphate and thiamine pyrophosphate (which are stored intracellularly). The transport of folates is uphill into the cell by the antiporter due to low concentration of folates inside the cell. 175 RFC has a higher affinity for reduced folates (Kₘ = 1-3 µM) and lower affinity for oxidized folates like folic acid (Kₘ = 200-400 µM). 176 RFC is ubiquitously expressed in normal and malignant cells in humans. The cancer cells are present in bone marrow, breast, lung, heart, small intestine and lymphocytes. 177-179

(b) Proton-Coupled Folate Transporter (PCFT, SLC46A1):

PCFT (Figure 27) is abundantly expressed in human tumors and is active under pH conditions associated with the tumor microenvironment. 180 It is a unidirectional symporter that transports folates with protons into the cells. 181 In the upper small intestine, the concentration of protons is high due to the activity of Na⁺/H⁺ exchanger and the high extracellular H⁺ concentration acts as the driving force for the PCFT symport into the cell. 182 PCFT functions optimally at pH 5.5. 181 As the pH increases the activity of the transporter decreases. 181 Unlike RFC, PCFT has an equal affinity for both folic acid and reduced folates. 182, 183 PCFT has 12 TMDs and both of its N-
and C-termini are present in the cytosol. The amino acid similarity between PCFT and RFC is 14%. The X-ray crystal structures for PCFT and RFC have not yet been resolved, which makes the binding interactions and the drug design challenging.

(c) Folate receptors (FRα, FRβ, FRγ):

Folate receptors and transporters and one-carbon metabolism play essential roles in the treatment of cancer. FRs are glycoproteins with high affinity for folates and are encoded by three different loci of FRα, FRβ, and FRγ. There is 70-80% amino acid homology among the FRs and have 245-257 amino acid residues. FRα and FRβ are glycosylphosphatidylinositol (GPI)-anchored cell surface glycoproteins and FRγ is a secretory protein. The FRα and β uptake is receptor-mediated endocytosis. FRα is expressed in epithelial cells of uterus, placenta, choroid plexus, retina and kidney. Moreover, it is expressed in cancers of epithelial origin- adenocarcinoma of ovary, cervix, uterus, kidney, lung, breast, bladder and pancreas. The expression of FRβ is in placenta, thymus, spleen, and malignancies of myelomonocytic origin.

![Figure 28. X-ray co-crystal structure of FRα with AGF183 (Pink) (PDB: 5IZQ, 3.6Å)](image)

visualized using Schrödinger Maestro 2020-1.
The X-ray crystal structures of FRα and FRβ have been resolved which demonstrates the co-crystal structure of a known antifolate AGF183 displayed in Figure 28.\textsuperscript{179,190} The 2-amino-4-oxo pyrimidine motif is commonly observed across folates and antifolates. The 2-NH\textsubscript{2} forms an ion-dipole interaction with the Asp81 side chain. The 3-NH interacts with the side chain of Ser174 via hydrogen bond interactions. The 4-oxo forms a hydrogen bond with the side chain of Arg103. The pyrrolo[2,3-\textit{d}]pyrimidine scaffold is stabilized by π-π stacking interactions with Tyr85 and Trp171. The L-glutamate side chain extends into a pocket composed of hydrophilic side chains. The α-COOH forms ion-dipole interactions with the Trp140 side chain and backbone NHs of Trp138 and Gly137. The γ-COOH forms ionic interactions with Lys136 and ion-dipole interactions with side chains of the Trp102 and Gln100.

Figure 29. X-ray co-crystal structure of FRβ with Pemetrexed (PMX, green) (PDB: 4KN2, 2.6 Å)\textsuperscript{141} visualized Schrödinger Maestro 2020-1.\textsuperscript{156}
The co-crystal structure of FRβ with MTX has been resolved, which is visualized in Figure 29. The stabilization of the 2,4-diamino scaffold is observed by π-π stacking interactions with Tyr101 and Trp187. The 2-NH\textsubscript{2} forms hydrogen bonding interactions with Ser190 side chain and the 4-NH\textsubscript{2} forms ion-dipole interaction with Asp97 side chain. The phenyl linker is also stabilized by π-π stacking interactions with Phe78 and Trp118. The \textit{L}-glutamyl NH forms a hydrogen bonding interaction with His151 backbone. The anionic side is oriented in a solvent exposed pocket. The α-COOH forms ion-dipole interactions with Trp154 and Trp156 side chain and backbone NH of Gly153. The γCOOH forms ionic interactions with Arg152 and ion-dipole interactions with side chains of Trp118 and Gln116.

A.3.3.2 Transporters for efflux of folates

In humans several ABC transporters exist where multidrug resistance proteins (MRP), MRP1-5 are capable of exporting folate derivatives.\textsuperscript{191} Another exporter of folates is breast cancer resistance protein (BRCP/ABCG2).\textsuperscript{192} Overexpression of these transporters in cancer cells can mediate the removal of (anti)folates from the cell.

A.3.3.3 Folates retention in the cell

Folates undergo folylpolyglutamylation following the uptake and this process is catalyzed by the enzyme folylpoly-γ-glutamate synthetase (FPGS).\textsuperscript{193} It catalyzes sequential addition of multiple equivalent of glutamic acid to the γ-carboxyl chain of the folate cofactors. As a result, there is an increased intracellular retention of these polyanions. There are two forms of FPGS: cytosolic and mitochondrial forms.\textsuperscript{167} Polyglutamylated folates are recognized as a much better
substrates for various folate-dependent enzymes.\textsuperscript{194} Folylpolyglutamylation process can be inverted by the enzyme $\gamma$-glutamyl hydrolase (GGH), which catalyze the hydrolysis of the $\gamma$-glutamyl tail of polyglutamylated folates.\textsuperscript{195}

**C.3.4 Antifolates and their clinical uses**

![Methotrexate (MTX)](image1)

![Raltitrexed (RTX, ZD1694)](image2)

![Pemetrexed (PMX, LY231514)](image3)

![Pralatrexate (PDX)](image4)

**Figure 30.** Clinically used antifolates

For over 60 years, antifolates have served important therapeutic roles as anticancer, antimicrobial and immunomodulatory agents.\textsuperscript{196} Aminopterin, the first antifolate, was well-known for cancer therapy.\textsuperscript{197} Aminopterin was replaced with methotrexate (MTX) in the 1950s due to its low therapeutic index.\textsuperscript{198} MTX is a DHFR inhibitor, which is taken up by cells via PCFT but mainly by RFC transport (Figure 26).\textsuperscript{162} Raltitrexate (RTX) (Tomudex\textsuperscript{®}) (Figure 30) is a potent inhibitor of TS (Ki = 62 nM) and is transported into cells by RFC and FR$\alpha$.\textsuperscript{199, 200} When compared to the monoglutamylated form the polyglutamylated form of RTX increases the inhibition for TS by 100-fold. PMX (Alimga\textsuperscript{®}) (Figure 30) is a TS inhibitor and is an excellent substrate for FPGS. The pentaglutamylated form of PMX displays an 84-fold increase in potency for TS, compared to the monoglutamylated form.\textsuperscript{201, 202} Pemetrexed (PMX) (Alimga\textsuperscript{®}) was introduced in the market for
treatment of mesothelioma and non-small cell lung cancer in 2004.\textsuperscript{82, 203} The pentaglutamylated form of PMX also shows moderate potency against GARFTase and AICARFTase enzymes, the monoglutamylated form is inactive against these two targets.\textsuperscript{201} As a result, TS is the primary target for PMX. Pralatrexate (PDX) (Folotyn\textsuperscript{®}) (Figure 30) is a DHFR inhibitor with an intracellular uptake through RFC.\textsuperscript{82, 203} In patients it has been approved with peripheral T-cell lymphoma.\textsuperscript{82, 203}

As a main mechanism of entry into the cell all the clinically used folates utilize RFC transport.\textsuperscript{196} These compounds cause dose-limiting toxicities (severe myelosuppression) due to their ubiquitous expression in RFC.\textsuperscript{204, 205} They do not have inherent selectivity for cancer cells. Polyglutamylation decreases when the concentration of intracellular folates (e.g., 5-formyl-THF) increases. As a result, when the concentration of intracellular folates increases the activity of PMX will decrease. Moreover, PMX-resistant cancers have been identified which were developed due to mutagenesis in the FPGS active site.\textsuperscript{206-208} Since polyglutamylation is responsible for increased potency, loss of FPGS activity can lead to drug-resistant cancer cell lines.\textsuperscript{207} Several studies have displayed reduced activity of such FPGS-dependent antifolates in cancer cell lines. These cell lines have low FPGS activity or have mutated FPGS incapable of binding to the antifolate.\textsuperscript{206, 207} Decreased activity of antifolates is observed with an increase in GGH activity which can cause resistance.\textsuperscript{209} Resistance can also be developed by the overexpression and mutations in the active site of DHFR or TS.\textsuperscript{206-208}

There is a critical need to develop agents where drug resistance and dose-limiting toxicities can be circumvented by (a) increased selectivity for transport via PCFT or FRs over RFC to eradicate or decrease dose-limiting toxicities; (b) ability to target two or more intracellular enzymes to decrease the development of resistance; and (c) absence of dependence on FPGS for activity against intracellular targets. Dies \textit{et al.}\textsuperscript{210} and Wang \textit{et al.}\textsuperscript{211} report 6-substituted
pyrrolo[2,3-d]pyrimidines with selectivity for transport through PCFT and FRs over RFC. These compounds showed potent GARFTase (inhibit purine synthesis) inhibition. This inhibition in GARFTase are mainly due to their monoglutamylated form and do not depend on FPGS.

**A.3.5 Inhibition of de novo purine synthesis**

![Diagram of purine nucleotide biosynthesis pathway](image)

**Figure 31.** De novo purine nucleotide biosynthesis pathway.\(^{212}\) (Modified from ref 212)

Purines, a coenzyme A (coA,) serve as building blocks of DNA and RNA, and as a component of ATP, cyclic adenosine monophosphate (cAMP), nicotinamide adenine dinucleotide (NAD).\(^{196}\) The synthesis of purines can occur through de novo synthetic pathway or salvage pathway.\(^{184}\) The de novo synthetic pathway is a 10-step folate-dependent pathway (from
phosphoribosyl pyrophosphate (PRPP) to AMP) (Figure 31). Free purine bases, which are derived from the turnover of nucleotides or from the diet, can be attached to PRPP to form purine nucleoside monophosphates. There are two types of phosphoribosyl transferases: adenine phosphoribosyl transferase (APRT) for AMP formation and hypoxanthine-guanine phosphoribosyl transferase (HGPRT) for IMP and GMP formation. Many enzymes required for salvages pathways get co-deleted in cancer cells and cancer cells remain dependent on the de novo synthesis for obtaining purines. Further, methylthioadenosine phosphorylase (MTAP), another salvage enzyme that releases adenine from methylthioadenosine is abundantly expressed in normal tissues and is co-deleted with CDKN2A in many tumors.

Table 3. GARFTase inhibition constants (Ki)

<table>
<thead>
<tr>
<th></th>
<th>X</th>
<th>n</th>
<th>Ar</th>
<th>Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGF23</td>
<td>NH</td>
<td>4</td>
<td>1,4-phenyl</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>AGF50</td>
<td>S</td>
<td>4</td>
<td>1,4-phenyl</td>
<td>1067 ± 271</td>
</tr>
<tr>
<td>AGF71</td>
<td>NH</td>
<td>4</td>
<td>2,5-thiophenyl</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>AGF94</td>
<td>NH</td>
<td>3</td>
<td>2,5-thiophenyl</td>
<td>68 ± 11</td>
</tr>
</tbody>
</table>
(a) GARFTase

Gangjee and coworkers have published 6-substituted pyrrolo[2,3-\textit{d}]pyrimidines which displayed GARFTase inhibition (Table 3). The reported compounds showed potent inhibition of GARFTase in their monoglutamylated form.

**Figure 32.** X-ray co-crystal structure of GARFTase and antifolate AGF150 (PDB: 4ZZ1, 1.35 Å). AGF150 is displayed in green and the substrate GAR is displayed in yellow. The co-crystal structure was visualized Schrödinger Maestro 2020-1.

The X-ray co-crystal structure of GARFTase and antifolate AGF150 (PDB: 4ZZ1, 1.35 Å) (Figure 32) can illustrate the binding mode of antifolates in GARFTase can be visualized by studying. The N1 of the pyrrolo[2,3-\textit{d}]pyrimidine scaffold forms a hydrogen bond with NH of the backbone of Leu899. The 2-NH$_2$ shows a hydrogen bond with backbone NHs of Ala947 and Glu948. The 3-NH also forms a hydrogen bond with the backbone NH of Ala947. The 4-oxo forms hydrogen bond with backbone NH of Asp951. The glutamate side chain is oriented in a solvent-
exposed pocket. The α-COOH forms an ionic bond with Arg871 and an ion-dipole interaction with the backbone NH of Ile898.

(b) AICARFTase

Adenine phosphoribosyl transferase (APRT) and adenosine kinase (AK) metabolize 5-Aminoimidazole-4-carboxamide (AICA) and AICAR to AICAR monophosphate (ZMP).\textsuperscript{184} Thus, this can circumvent the reaction catalyzed by GARFTase. Moran and coworkers\textsuperscript{218} reported an interesting find on the effect of PMX on AICARFTase. Inhibition of AICARFTase causes accumulation of its substrate- AICAR (ZMP). The accumulated ZMP causes activation of AMP-dependent protein kinase (AMPK), the activation of AMPK causes inhibition of the mechanistic target of rapamycin (mTOR) as well as direct phosphorylation of the raptor component of mammalian target of rapamycin complex (mTORC1 complex). Thus, it inhibits cell growth through two distinct pathways.

\textbf{Figure 33.} X-ray co-crystal structure of AICARFTase with bicyclic classical antifolate compound (PDB: 1P4R, 2.55 Å).\textsuperscript{219} The ligand and AICAR are displayed in cyan and magenta color, respectively. The co-crystal structure was Schrödinger Maestro 2020-1.\textsuperscript{156}
To illuminate the binding mode of compounds in AICARFTase the X-ray cocrystal structure of AICARFTase with a bicyclic classical antifolate has been revealed (Figure 33).\textsuperscript{219} Stabilization of the bicyclic scaffold occurs by π-π stacking interaction with Phe544. The 2-NH\textsubscript{2} displays hydrogen bond with the backbone carbonyl of Asn489 and an ion-dipole interaction with the side chain of Asp546. The 3-NH also displays ion-dipole interaction with the side chain of Asp546. The 4-oxo group forms a hydrogen bond with the side chain of Asn547. The sulfone in the linker shows hydrogen bonding interaction with backbone NH of Arg541, the side chain of Asn431 and an ion-dipole interaction with Lys266. The glutamate side chain is oriented in a hydrophilic, solvent-exposed pocket.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure34.png}
\caption{5-substituted pyrrolo[2,3-\textit{d}]pyrimidine compounds 10-13 with inhibitory activity against GARFTase and AICARFTase.\textsuperscript{220, 221}}
\end{figure}

The 5-substituted pyrrolo[2,3-\textit{d}]pyrimidines 10-13 were reported by Gangjee and coworkers\textsuperscript{220, 221} (Figure 34), which exhibited inhibition of both GARFTase and AICARFTase.
This dual inhibition attacks the purine biosynthetic pathway at two distinct sites and could overcome tumor resistance developed at a single site. Such a multitarget agent will be effective for drug-resistant tumors and will be advantageous in anti-folate drug discovery.

A.3.6 Inhibitors of serine hydroxymethyl transferase enzyme

One-carbon metabolism incorporates a broad range of biosynthetic reactions that can occur in the cytoplasm and the mitochondria. These are essential for maintaining cellular homeostasis and supports multiple physiological functions. The reactions include biosynthesis (purines and thymidine), amino acid homeostasis (glycine, serine, and methionine), epigenetic maintenance, and redox defense. One carbon metabolic reactions are compartmentalized in both eukaryotic cells and across organs. Mitochondrial 1C reactions are crucial, both for producing 1C units that are exported to the cytosol and for making additional products, including glycine and NADPH. Serine is a key metabolic source for producing one-carbon (1C) units in mammalian cells. Two serine hydroxymethyl transferase (SHMT) enzymes, SHMT1 and SHMT2, break down serine into glycine and methylene-tetrahydrofolate (THF) in the cytosol and mitochondria, respectively. The latter serine catabolite feeds into cellular 1C pool, and either directly participates in thymidine synthesis or indirectly in purine or methionine synthesis after its oxidative or reductive conversion to formyl- or methyl-THF. Because the 1C-derived products are key anabolic building blocks, sustaining the 1C pool is vital for cellular proliferation and is required for a number of physiological and pathophysiological processes ranging from stem cell renewal to cancer progression. Consistent with their critical roles in supporting cell proliferation, SHMTs are highly active in many rapidly-growing cancer cells and are important molecular targets for cancer intervention.
Figure 35. C1 metabolism is compartmentalized in the cytosol and mitochondria.\textsuperscript{229,230} (Modified from 229 and 230)

The entry of folates to the cell through the plasma membrane is facilitated by folate transporters, PCFT, RFC and mitochondria via SLC25A32 (Figure 35). One carbon units incorporated in various folate metabolism cofactors are primarily derived from serine catabolism occurring in mitochondria.\textsuperscript{222} Thus, serine catabolism plays an indispensable role in cancer growth.\textsuperscript{222,224} Serine is catabolized in mitochondria and synthesized in the cytosol by a set of enzymes that allow not only for parallel metabolic processes but also for a complete oxidative/reductive cycle.\textsuperscript{222} In mitochondria, serine is metabolized to formate via three enzymes: a) SHMT2, b) methylene tetrahydrofolate dehydrogenase 2 (MTHFD2), and c) methylene tetrahydrofolate dehydrogenase 1 like (MTHFD1L). In the cytosol, serine is synthesized via a) methylene tetrahydrofolate dehydrogenase 1 (MTHFD1) and b) SHMT1. The cycle that
catabolizes serine in mitochondria and synthesizes serine in cytosol is thermodynamically driven by the difference in electrochemical potential between NADH (NADPH) and cytosolic NADPH.

There is a continuous need for 1C supply in rapidly proliferating cells such as cancer cells which results in the overexpression of SHMT2 and MTHFD2. The 1C unit generated from the serine catabolism in the mitochondria is exported to cytosol as formate. The MTHFD1 incorporates the formate into the THF to form 10-formylTHF. Subsequently, by a series of reactions catalyzed by GARFTase, AICARFTase and thymidylate synthase (TS), the 1C unit is used for nucleotide synthesis. SHMT2 (or MTHFD2) knockout (KO) cells are viable and tumorigenic (albeit with decreased growth rates) in nutrient-rich conditions, as the reversal of cytosolic SHMT1 (serine-to-glycine) provides sufficient C1 units to sustain some level of de novo nucleotide biosynthesis. Additionally, SHMT1 does not generate sufficient glycine for protein, nucleotide and glutathione biosynthesis, rendering both SHMT2 and MTHFD2 KO cells as glycine auxotrophs. Simultaneous inhibition of cytosolic and mitochondrial SHMT enzyme is necessary to achieve the cytotoxic effect. Gangjee and coworkers have recently reported pyrrolo[3,2-d]pyrimidine antifolates which inhibit both SHMT1 and SHMT2 along with de novo purine biosynthesis pathway and are transported selectively into tumor cell. This resulted in outstanding in vivo efficacy in a pancreatic cancer model. These compounds are exciting prototypes for dual-targeting mitochondrial and cytosolic C1 metabolism for cancer, with significant promise for overcoming resistance to current anticancer therapies.
II. CHEMICAL REVIEW

The chemistry related to the work carried out is reviewed in this section and includes synthetic approaches to the following heterocyclic ring systems:

B.1. Thieno[2,3-\textit{d}]pyrimidines
B.2. Pyrrolo[3,2-\textit{d}]pyrimidines
B.3. Pyrazolo[4,3-\textit{d}]pyrimidines
B.4. Pyrido[2,3-\textit{d}]pyrimidines
B.5. Pyrrolo[2,3-\textit{d}]pyrimidines
B.6 Name Reactions

B.1. Synthesis of thieno[2,3-\textit{d}]pyrimidines

Scheme 1. Retro-synthetic analysis of thieno[2,3-\textit{d}]pyrimidines

The synthetic strategy for the construction of thieno[2,3-\textit{d}]pyrimidines includes two categories (Scheme 1):

1. From thiophene precursors (Route A)
2. From pyrimidine precursors (Route B)
1. From thiophene precursors (Route A)

1.1 Condensation to obtain 2-amino-5,6-disubstituted thieno[2,3-\(d\)]pyrimidines

**Scheme 2.** Synthesis of thieno[2,3-\(d\)]pyrimidine 18

\[
\begin{array}{c}
\text{X} \quad \text{R}^1 \quad \text{R}^2 \quad \text{H}_2\text{N} \quad \text{NH} \quad \text{R} \quad \text{NH}_2 \\
\text{14 X = CN,} \\
\text{15 X = COOR,} \\
\text{16 X = CHO} \\
\end{array}
\]

Reagents and conditions: a) DMSO\(_2\), 140 °C

2-Amino-5,6-disubstituted thieno[2,3-\(d\)]pyrimidines 18 (Scheme 2) were reported to be synthesized via cyclocondensation of appropriate thiophenes 14-16 with an amidine derivatives 17\(a\)-17\(b\),\(^{235}\) which can be guanidine (R = NH\(_2\)) or chloroformamidine hydrochloride (R = Cl). The nature of the X substitution in thiophene 14-16 determines the substitution pattern at 4-position (Y at the C4-position) in 18: when X = CN, COOR or CHO, the cyclization reaction affords 18 with Y = NH\(_2\), OH or H respectively.

**Scheme 3.** Synthesis of thieno[2,3-\(d\)]pyrimidine from formamide.

\[
\begin{array}{c}
\text{O} \quad \text{C} \quad \text{H}_2\text{N} \quad \text{NH} \quad \text{R} \quad \text{NH}_2 \\
\text{19} \\
\end{array}
\]

Reagents and conditions: a) 170° C, 12h\(^{236}\)
The synthesis of thieno[2,3-\(d\)]pyrimidin-4(3\(H\))-one 21 (Scheme 3) was reported by Fyfe et al.,\textsuperscript{236} from methyl 2-aminothiophene-3-carboxylate (19) and formamide 20 at 170 °C under a nitrogen atmosphere.

**Scheme 4.** Synthesis of thieno[2,3-\(d\)]pyrimidine from thioamide.

Reagents and conditions: a) Microwave irradiation\textsuperscript{237}

The thioamide 23 and amino-thiophene-carboxylates 22 were used as starting materials for the construction of thieno[2,3-\(d\)]pyrimidin-4-amines 24\(a\)-24\(e\) following literature method (Scheme 4).\textsuperscript{237} The thioamide 23 can be synthesized from the corresponding amide by reaction with Lawesson’s reagent.\textsuperscript{237} In this context, the preparation of thienopyrimidin-4-ones, which usually requires very vigorous reaction conditions and prolonged reaction times, can be achieved starting from the corresponding thioamides 23, amino-thiophene-carboxylates 22 as hydrochlorides, without solvent and under microwave irradiation. In a typical general procedure, twenty 4 mL open vials containing a homogenized mixture of the corresponding thioamide 23 (3 mmol) and amino-thiophene-carboxylates 22, as hydrochlorides (1 mmol), were inserted into a Teflon disc and irradiated at 550 Watt power for 3.5 minutes.\textsuperscript{237}
Scheme 5. Synthesis of thieno[2,3-d]pyrimidine from nitrile and HCl gas.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{C} & \quad \text{H}_2\text{C} \quad \text{C} \\
\text{S} & \quad \text{CN} \\
\end{align*}
\]

Reagents and conditions: a) HCl (gas), Dioxane, r.t., 2-3 h.

Synthesis of 27 were described by Shishoo et al.,\textsuperscript{238} (Scheme 5) which involves bubbling dry HCl gas through a stirred solution of 25 and the corresponding nitrile 26 in dioxane at room temperature for 2–3 hours (until saturation). Then stirring the mixture was done at room temperature for a further 12 h period. This procedure yielded the corresponding thieno pyrimidinones 27, which were simply isolated and purified by trituration with diethyl ether.\textsuperscript{237}

Scheme 6. Synthesis of thieno[2,3-d]pyrimidine from chloroacetonitrile

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \\
\text{C} & \quad \text{H}_2\text{C} \quad \text{C} \\
\text{S} & \quad \text{CN} \\
\end{align*}
\]

Reagents and conditions: a) sulfur, morpholine, ethyl cyanoacetate, rt, 18 h (b) chloroacetonitrile, HCl (g), 50 °C to reflux, 4–24 h\textsuperscript{236}

An equimolar mixture of powdered sulfur and morpholine was stirred until total dissolution of the sulfur (Scheme 6). Ethyl cyanoacetate and butan-2-one 28 were added to the reactional mixture, which was stirred at room temperature for 18 h to get cyclized ethyl 2-amino-4,5-
dimethylthiophene-3-carboxylate 29. Compound 30 was synthesized by reacting 29 and chloroacetonitrile in 1,4-dioxane and heated to 50 °C, and passed with dry HCl gas until the starting material was disappeared in 3–24 h or a precipitate is formed.\textsuperscript{256}

**Scheme 7.** Synthesis of thieno[2,3-\textit{d}]pyrimidine from urea.

\[
\begin{align*}
\text{Ph} & \quad \text{C} \\
\text{H}_2\text{N} & \quad \text{S} \\
\text{R}_1 & \quad \text{R}_2 \\
\end{align*}
\]
\[
+ \quad \begin{align*}
\text{H}_2\text{N} & \quad \text{C} \\
\text{NH}_2 & \quad \text{NH}_2 \\
\end{align*}
\]
\[
\text{N} \\
\text{Ph} \\
\text{CH}_3 \\
\text{CH}_3 \\
\text{N} \\
\text{N} \\
\end{align*}
\]

31 32 33

Reagents and conditions: a) 170 °C, 2 h.

Ishikawa and coworkers\textsuperscript{239} reported a novel synthesis of 2-amino-4-phenyl substituted thieno[2,3-\textit{d}]pyrimidines 33 where the condensation of aminocarbonyl thiophenes 31 and urea 32 was done to afford thieno[2,3-\textit{d}]pyrimidine 33 (Scheme 7).

**Scheme 8.** Synthesis of 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidin-4(3\textit{H})-one from formamide.

\[
\begin{align*}
\text{C} & \quad \text{C} \\
\text{N} & \quad \text{NH} \\
\end{align*}
\]
\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{S} & \quad \text{N} \\
\end{align*}
\]

34 35

Reagents and conditions: a) formamide, 170 °C, 12 h\textsuperscript{236}
Ethyl-2-amino-4,5,6,7-tetrahydrobenzothiophene-3-carboxylate (34) (Scheme 8) was taken up in formamide and heated to 170 °C under a nitrogen atmosphere to obtain 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one 35.236

**Scheme 9.** Synthesis of 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one from chloroacetonitrile

![Chemical structure](image)

Reagents and conditions: a) morpholine, rt, 12h b) chloroacetonitrile, 1,4-dioxane, HCl(g) (Kipp’s apparatus), 50°C, 2 h236

Sulfur 37 and morpholine was stirred until total dissolution of the sulfur (Scheme 9). Cyclohexanone 36 and ethyl cyanoacetate 38 were added to the reactional mixture, which was stirred at room temperature for ~12 h to get cyclized ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate 34. Compound 39 was synthesized by reacting 34 and chloroacetonitrile in 1,4-dioxane and heated to 50 °C, and passed with dry HCl gas (Kipp’s apparatus) until the starting material was disappeared in 3–24 h or a precipitate formed.236

**B.2. Synthesis of pyrrolo[3,2-d]pyrimidines**

Elliott et al.,240 published a method (Scheme 10) of synthesizing 7-Substituted Pyrrolo[3,2-d]pyrimidine 45. Aldehydes 40 and 3,3-dimethoxypropionitrile were mixed together and added to a solution of NaOMe in MeOH to synthesize 41 followed by treatment with 6N HCl to deprotect the acetal group and provide 42 (Scheme 12). Catalytic hydrogenation of 42 in MeOH followed
by condensation of the resulting 43 with diethylamino malonate gave the required enamine 44. On treatment with NaOMe in MeOH at room temperature, 44 was smoothly converted to 45. Removal of the MeOH and addition of water gave essentially pure pyrrole (45a) in 57% yield, and thus, this sequence obviated the need for purification by column chromatography. Other pyrroles (45b-45i) behaved similarly.

**Scheme 10.** Synthesis of 7-Substituted pyrrolo[3,2-d]pyrimidine from aldehydes and 3,3-dimethoxypropionitrile.

Reagents and conditions: a) NCCH\(_2\)CH(OCH\(_3\))\(_2\), NaOMe; b) 6 N HCl; c) H\(_2\), Pd/ C; d) H\(_2\)NCH(COOEt)\(_2\); e) NaOMe, MeOH.

**Scheme 11.** Synthesis of pyrrolo[3,2-d]pyrimidines from methyl pyrrole 2-carboxylate and acid chlorides.

Reagents and conditions: a) AlCl\(_3\), appropriate acid chloride, DCM, 0 °C- r.t., 16 h; b) F\(_3\)CCOOH, Et\(_3\)SiH, r.t., overnight.
Regioselective Friedel-Crafts acylation\textsuperscript{241} of commercially available methyl pyrrole 2-carboxylate (46) using the appropriate acid chlorides and aluminium trichloride gave methyl esters 47 in 63-74\% yields. Reduction\textsuperscript{242} of 47 with \(\text{Et}_3\text{SiH}\) and \(\text{F}_3\text{CCOOH}\) yielded 48 (Scheme 11).

**Scheme 12.** Synthesis of 2-alkyl-4-oxo-pyrrolo[3,2-\(d\)]pyrimidines from ethyl 3-amino-5-substituted-1\(H\)-pyrrole-2-carboxylate

\[
\begin{align*}
\text{AcO} & \quad \text{R} \quad \text{H}_2\text{N} \quad \text{H} \\
\text{O} & \quad \text{N} \quad \text{C} \quad \text{N} \quad \text{N} \\
\text{H}_2\text{N} & \quad \text{Alkyl} \\
\text{R} & \quad \text{Alkyl} \\
\text{49} & \quad \text{50}
\end{align*}
\]

Reagents and conditions: a) (i) Alkynitrile, HCl(g); (ii) NaOH, EtOH

Pyrrolo[3,2-\(d\)]pyrimidines 50 with 2-alkyl substituents are obtained by treatment of 5-substituted pyrrole 49 (Scheme 12) with an alkyl nitriles in anhydrous hydrochloric acid followed by heating with sodium hydroxide.\textsuperscript{243} For \(R = \text{methyl}\), the yield was 53\%.

**Scheme 13.** Synthesis of 3\(H\)-pyrrolo[3,2-\(d\)]pyrimidin-4(5\(H\))-one from ethyl 3-amino-1\(H\)-pyrrole-2-carboxylate

\[
\begin{align*}
\text{AcO} & \quad \text{R}_1 \quad \text{R}_2 \quad \text{H}_2\text{N} \\
\text{O} & \quad \text{N} \quad \text{C} \quad \text{N} \quad \text{N} \\
\text{H}_2\text{N} & \quad \text{R}_1 \\
\text{51} & \quad \text{52}
\end{align*}
\]

Reagents and conditions: a) formamidine acetate, EtOH

Pyrrolo[3,2-\(d\)]pyrimidines with 2-\(H\) 52 are obtained by cyclocondensation of substituted ethyl 3-amino-1\(H\)-pyrrole-2-carboxylate 51 (Scheme 13) with formamidine acetate in refluxing ethanol.\textsuperscript{244}

Reagents and conditions: a) S=C=NCOOC₂H₅, HCl; b) aq. NaOH

Pyrrolo[3,2-d]pyrimidine 54 with 2-sulfanyl substituent can be obtained by treatment of 2-amino-6-phenyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one 53 (Scheme 14) with an ethyl isothiocyanatoformate and hydrochloric acid in benzene at reflux followed by heating with aqueous sodium hydroxide.²⁴⁵

B.3. Pyrazolo[4,3-d]pyrimidines

Scheme 15. Synthesis of 2,4-dichloro pyrazolo[4,3-d]pyrimidines from methyl-pyrazolo-carboxylic acids 55

Reagents and conditions: a) (i) fuming HNO₃, H₂SO₄, 0°C, (ii) SOCl₂, NH₄OH, 0°C to r.t., (iii) 10% Pd/C, EtOH, 60 psi H₂, over all 46-51% yield; b) CDI, ACN, reflux, 98%; c) POCl₃, N,N-diethylaniline, 130°C, 60-72%.²⁴⁶
The pyrazolo intermediates 56a and 56b (Scheme 15) were prepared following a literature method\textsuperscript{246} from readily available 1-methyl-1\textit{H}-pyrazole-5-carboxylic acid 55a and 1,3-dimethyl-1\textit{H}-pyrazole-5-carboxylic acid 55b, respectively. Pyrimidinediones 57a-57b were synthesized by treating 56a-56b and CDI in reflux condition in ACN. Chlorination was done by treatment of 57a-57b with phosphoryl chloride in the presence of \(N, N\)-diethylaniline to provide dichloropyrimidines 58a-58b in 60-72\% yield (Scheme 15).

**Scheme 16.** Synthesis of pyrazolo[4,3-\textit{d}]pyrimidines from 4-amino-3-methyl-1\textit{H}-pyrazole-5-carboxamide with thiourea.

Reagents and conditions: a) 190-210 °C

Robins \textit{et al.}\textsuperscript{247} (Scheme 16) accomplished the desired product 61 by the cyclization of 4-amino-3-methyl-1\textit{H}-pyrazole-5-carboxamide 59 with thiourea 60 under reflux conditions.

**Scheme 17.** Synthesis of 7-hydroxypyrazolo[4,3-\textit{d}]pyrimidine from 4-amino-1\textit{H}-pyrazole-5-carboxamide with formamide.

Reagents and conditions: a) \(H_2O\), 1h.
Robins et al., treated 4-amino-1H-pyrazole-5-carboxamide 62 with formamide 63 under boiling conditions to afford 7-hydroxypyrrozolo[4,3-d]pyrimidine 64 as a target molecule (Scheme 17).

**Scheme 18.** Synthesis of 3-methyl-7-thioxo-1,4,6,7-tetrahydro-5H-pyrazolo[4,3-d]pyrimidin-5-one from 4-amino-3methyl-1H-pyrazole-5-carbothioamide with urea.

![Diagram of Scheme 18]

Reagents and conditions: a) reflux

Long and co-workers introduced a fusion reaction by ring annulation of 4-amino-3methyl-1H-pyrazole-5-carbothioamide 65 with urea 66 to achieve 3-methyl-7-thioxo-1,4,6,7-tetrahydro-5H-pyrazolo[4,3-d]pyrimidin-5-one 67 (Scheme 18).

**Scheme 19.** Synthesis of pyrazolo[4,3-d] pyrimidinedione from 5-carbamoyl-1H-pyrazole-4-carbonyl azide.

![Diagram of Scheme 19]

Reagents and conditions: a) toluene, reflux
Curtius rearrangement\[^{249}\] was carried out in boiling toluene to generate pyrazolo[4,3-\(d\)]pyrimidinedione 69 as a final compound (Scheme 19) from 5-carbamoyl-1\(H\)-pyrazole-4-carbonyl azide 68.

**Scheme 20.** Synthesis of pyrazolo[4,3-\(d\)]pyrimidine from 4-nitro-1\(H\)-pyrazole-5-carboxamide.

![Scheme 20](image)

Reagents and conditions: a) DBAD/Ph\(_3\)P; b) H\(_2\), Pd/C; c) CDI; d) POCl\(_3\)

Tollefson and co-workers\[^{250}\] carried out a Mitsunobu reaction (Scheme 20) of the alkoxy ethanol (71) with 4-nitro-1\(H\)-pyrazole-5-carboxamide (70) to afford the \(N\)-alkylated pyrazole (72). The nitro group in compound 72 was reduced to the amine 73 via hydrogenation. Cyclization of compound 73 to the dione 75 was accomplished by reaction with carbonyldiimidazole (CDI, 74). Finally, chlorination of 75 using phosphorus oxychloride afforded the required pyrazolopyrimidine dichloride 76.
**Scheme 21.** Synthesis of pyrazolo[4,3-d]pyrimidine from ethyl 4-amino-1-(4-methoxyphenyl)-1H-pyrazole-3-carboxylate and 1,1,1-triethoxyethane

[Chemical structure image]

Reagents and conditions: a) NH₄OAc, MW.

Lenzi et al.²⁵¹ attempted the cyclization reaction of ethyl 4-amino-1-(4-methoxyphenyl)-1H-pyrazole-3-carboxylate 77 with 1,1,1-triethoxyethane 78 in presence of ammonium acetate to afford 79 under microwave irradiation conditions (Scheme 21).

**Scheme 22.** Synthesis of pyrazolo[4,3-d]pyrimidine with potassium hydroxide or potassium tert-butoxide.

[Chemical structure image]

Reagents and conditions: a) KOH, EtOH or t-BuOK, THF

Brantenko and co-workers²⁵² attempted intramolecular cyclization reaction of 80 by treating with potassium hydroxide in EtOH or potassium tert-butoxide in THF to afford 81 (Scheme 22).
Scheme 23. Synthesis of pyrazolo[4,3-\textit{d}]pyrimidine from 4-amino-1-phenyl-1\textit{H}-pyrazole-3-carbonitrile and ethyl (E)-3-(4-methoxyphenyl)acrylimidate hydrochloride.

![Chemical structure](image)

Reagents and conditions: a) NH$_4$OAc, MW, 150 °C

Squarcialupi \textit{et al.},$^{253}$ reacted 4-amino-1-phenyl-1\textit{H}-pyrazole-3-carbonitrile (82) with ethyl (E)-3-(4-methoxyphenyl)acrylimidate hydrochloride 83 to attain 84 in presence of ammonium acetate under microwave conditions (Scheme 23).

Scheme 24. Synthesis of pyrazolo[4,3-\textit{d}]pyrimidine from 6-(bromomethyl)-1,3-dimethyl-5-nitropyrimidine-2,4(1\textit{H}, 3\textit{H})dione and methylamine.

![Chemical structure](image)

Reagents and conditions: a) EtOH, reflux, 4h.

Senda \textit{et al.},$^{254}$ have accomplished the desired molecule 87 by reacting 6-(bromomethyl)-1,3-dimethyl-5-nitropyrimidine-2,4 (1\textit{H}, 3\textit{H}) dione 85 with methylamine 86 in ethanol under reflux conditions (Scheme 24).
B.4. Pyrido[2,3-\textit{d}]pyrimidines

**Scheme 25.** Synthesis of pyrido[2,3-\textit{d}]pyrimidines from 2,4,6-triaminopyrimidine and bromomalondialdehyde

<table>
<thead>
<tr>
<th>R</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>85</td>
</tr>
<tr>
<td>4'-OMe</td>
<td>88</td>
</tr>
<tr>
<td>2'-OMe</td>
<td>56</td>
</tr>
<tr>
<td>4'-Me</td>
<td>93</td>
</tr>
<tr>
<td>4'-i-Pr</td>
<td>91</td>
</tr>
<tr>
<td>4-NO(_2)</td>
<td>60</td>
</tr>
</tbody>
</table>

Reagents and conditions: a) (i) BrCH(CHO)\(_2\), HCl, EtOH, 30 min; (ii) PivCl, pyridine, reflux; b) p-Toluidine, Pd\(_2\)dba\(_3\), LiHMDS, X-Phos, toluene, 100 °C

Gangjee \textit{et al.},\(^{154}\) reported method of synthesizing pyrido[2,3-\textit{d}]pyrimidine from 2,4,6-triaminopyrimidine \(88\) and bromomalondialdehyde in acidic conditions (Scheme 25). A further protection of the 2,4-diamino-6-bromopyrido[2,3-\textit{d}]pyrimidine \(89\) with the pivaloyl group increased solubility and facilitated purification of the final compound. Under these conditions, bis pivaloyl derivative \(89\) was obtained in a global yield of 39%. Buchwald–Hartwig aminations of \(89\) afforded \(90\) in 56-93% yields.

**Scheme 26.** Synthesis of pyrido[2,3-\textit{d}]pyrimidines from pyrido dimethyl amidine and substituted phenyl sulfanyl aniline.

Reagents and conditions: a) AcOH, reflux.
Krueger et al.\textsuperscript{255} investigated the synthesis pyrimidine derivatives in Scheme 26. The isopropyl pyrido[2,3-\textit{d}] pyrimidine analog 93 was constructed by cyclization of pyrido dimethyl amidine 91 with the substituted phenyl sulfanyl aniline 92 in moderate yield 10-54%.

**Scheme 27.** Synthesis of pyrido[2,3-\textit{d}]pyrimidines from 4-aminopyrimidine-5-carbaldehyde.

\[
\begin{align*}
\text{N} & \text{CHO} \quad \text{ArCOMe} \quad a \quad \text{N} \text{Py} \quad \text{Ar} \\
94 & \quad 95 & \quad 96
\end{align*}
\]

Reagents and conditions: a) K\textsubscript{2}CO\textsubscript{3}, acetone, r.t.

Rao group\textsuperscript{256} investigated the synthesis of 7-substituted pyrido[2,3-\textit{d}]pyrimidines in Scheme 27. The 7-substituted pyrido[2,3-\textit{d}] pyrimidine 96, easily obtained from 4-aminopyrimidine-5-carbaldehyde 94 and various aromatic or heteroaromatic ketones 95, in 78-85% yield.

**Scheme 28.** Synthesis of pyrido[2,3-\textit{d}]pyrimidines from 2,4,6-triaminopyrimidine and \textit{\beta}-ketoester.

\[
\begin{align*}
\text{MeC} & \quad \text{O} \quad \text{Me} \quad \text{NH}_{2} \quad \text{NH}_{2} \quad \text{NH}_{2} \quad \text{Me} \quad \text{Me} \quad \text{Ar} \\
97 & \quad 88 & \quad 98 & \quad 99
\end{align*}
\]

Reagents and conditions: a) NMP, 180 °C, 47%; b) (i) (COCl\textsubscript{2}), DMF, 70%; (ii) HCO\textsubscript{2}Na, 5% Pd/C, 97%; (iii) NIS, TFA, 70%

Rosowsky et al.,\textsuperscript{257} described the synthesis of pyrido[2,3-\textit{d}]pyrimidine 98 (Scheme 28) which was achieved by reaction of 2,4,6-triaminopyridine 88 and \textit{\beta}-ketoester 97, affording 98 in 47% yield. Derivative 99 obtained from 98 via a sequence of reactions described in Scheme 34.
Scheme 29. Synthesis of pyrido[2,3-\(d\)]pyrimidines from 2-amino-3-cyano-4trifluoromethyl-6-phenyl-pyridine and guanidine, thiourea, and urea.

Reagents and conditions: a) guanidine/thiourea/urea, C\(\text{2H}_5\text{ONa/EtOH, reflux 48–50 h.}\)

Chandrakaran et al.,\(^{258}\) reported a method of synthesizing substituted pyrido[2,3-\(d\)]pyrimidines (Scheme 29) from the precursor 2-amino-3-cyano-4trifluoromethyl-6-phenyl-pyridine 100 on a cyclocondensation reaction with guanidine, thiourea, and urea under basic conditions (sodium ethoxide) which resulted in the formation of 101, 102, and 103, respectively.

Scheme 30. Synthesis of pyrido[2,3-\(d\)]pyrimidines from 3-amino-2-methylacrylaldehyde and pyrimidine-2,4,6-triamine

Reagents and conditions: a) Glacial acetic acid, piperidine acetate\(^{259}\)

Gangjee et al.,\(^{259}\) reported the synthesis of 6-methylpyrido[2,3-\(d\)]pyrimidine-2,4-diamine in depicted in Scheme 30. To a solution of 2,4,6-triaminopyrimidine 88 in glacial acetic acid containing catalytic amount of piperidine acetate was added to powdered 3-amino-2-methylacrylaldehyde 104 at 60 °C. The mixture was refluxed and after the reaction the solution
was neutralized to pH 7 with concentrated ammonium hydroxide which afforded a yellow precipitate of 105.

**Scheme 31.** Synthesis of pyrido[2,3-\(\text{d}\)]pyrimidines from 2-nitromalonaldehyde and pyrimidine-2,4,6-triamine

Reagents and conditions: a) (i) HCl, EtOH, reflux, 10 min; (ii) NH\(_4\)OH, H\(_2\)O, neutralized; b) H\(_2\), Ni, rt, 35 psi; c) AcOH

Gangjee et al.\(^{260}\) reported method of synthesizing pyrido[2,3-\(\text{d}\)]pyrimidine (Scheme 31) from 2-nitromalonaldehyde 106 and pyrimidine-2,4,6-triamine 88. In acidic condition 2-nitromalonaldehyde 106 and pyrimidine-2,4,6-triamine 88 were refluxed in EtOH. *In situ* reduction of 107 with Raney Ni afforded the amino intermediate 108 which was condensed without isolation with benzaldehydes 109 to afford the target compounds 110 in 50 – 58% yield.

**B.5. Synthesis of pyrrolo[2,3-\(\text{d}\)]pyrimidines**

**Scheme 32.** Synthesis of pyrrolo[2,3-\(\text{d}\)]pyrimidines from 6-amino-2-(dimethylamino)pyrimidin-4-(3\(H\))-one

Reagents and conditions: a) *n*-butanol, reflux
Gangjee et al.\textsuperscript{261} reported the synthesis of 115 (Scheme 32) by the condensation of an aqueous solution of α-chloroacetone 111 in presence of NaOAc in 70% yield. Linz et al.\textsuperscript{262} also reported the synthesis of a series of pyrrolo[2,3-\textit{d}]pyrimidines from 6-amino-2-(dimethylamino)pyrimidin-4(3\textit{H})-ones 112 (Scheme 32). Compound 112 on treatment with α-chloroacetone 113 or α-chloroacetaldehyde 114 provided the corresponding pyrrolo[2,3-\textit{d}]pyrimidines 115 or 116 in 68% and 75% yields respectively.

**Scheme 33.** Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines using Fisher-Indole reaction

![Scheme 33](image)

Reagents and conditions: a) methyl 4-(4-oxopentyl)benzoate, 2-methoxyethanol; b) \textit{Ph}_2\textit{O}

Taylor \textit{et al.}\textsuperscript{263} synthesized methyl 4-(2-(2-amino-6-methyl-4-oxo-4,7-dihydro-3\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidin-5-yl)ethyl)benzoate 119 (Scheme 33) by a Fisher-Indole approach. Reaction of 2-amino-6-hydrazinylpyrimidin-4(3\textit{H})-one, 117 with one equivalent of methyl 4-(4-oxopentyl)benzoate in 2-methoxyethanol under reflux afforded the requisite intermediate 118, methyl (\textit{Z})-4-(4-(2-amino-6-oxo-1,6-dihydropyrimidin-4-\textit{y}l)hydrazineylidene)pentyl)benzoate. The cyclization was accomplished by thermolysis of 118 in refluxing diphenyl ether to regioselectivity afford the pyrrolo[2,3-\textit{d}]pyrimidine 119.
Scheme 34. Synthesis of 2-amino-pyrrolo[2,3-d]pyrimidines from 4-substituted 2,6-diamino pyrimidines

Reagents and conditions: a) DMF, 60°C, 4 d

Khalaf et al. synthesized pyrrolopyrimidines 122-123 (Scheme 34) where pyrimidine substrate, 2,6-diamino-4-oxo 120 were reacted with diaryl bromoketones 121 with DMF at 60°C.

Scheme 35. Synthesis of 2-amino-pyrrolo[2,3-d]pyrimidines from 2-bromo-1,1-diethoxyethane

Reagents and conditions: a) malononitrile, K₂CO₃, DMF, 50°C, 17–18 h; (b) thiourea, potassium tert-butoxide, EtOH, reflux, 16–17 h; r.t., 1–2 h; (c) 5 N HCl, H₂O, 10 N NaOH, 50°C, 50 min

El-Gamal and Oh synthesized the intermediate 2-(2,2-diethoxyethyl)malononitrile 127 (Scheme 35) from 2-bromo-1,1-diethoxyethane 124 with malononitrile by heating in N,N-dimethylformamide (DMF) in the presence of base K₂CO₃. Synthesis of 4,6-diamino-5-(2,2-diethoxyethyl)pyrimidine-2-thiolate 126 was carried out by refluxing the geminal dicyano 125 with thiourea in the presence of a stronger base potassium tert-butoxide. Cyclization to 126 could be achieved by neutralization of the thiol potassium salt 125 using 5 N aqueous HCl followed by heating with 10 N aqueous NaOH to afford 7H-pyrrolo[2,3-d] pyrimidin-4-amine 127.
Scheme 36. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from 4-chloro-5-iodo-2,6-dimethylpyrimidine

\[
\begin{align*}
\text{Reagents and conditions: a) Pd(OAc)}_2; & \quad \text{b) PdCl}_2; \quad \text{c) NaN}_3; \quad \text{d) heat or UV} \\
\text{Kondo and coworkers}^{266} & \text{ reported the synthesis of pyrrolo[2,3-\textit{d}]pyrimidine 133 via a} \\
\text{photoinduced or thermal cyclization of 4-azidopyrimidines 132 containing an olefinic} \\
\text{functionality at the 5-position (Scheme 36). Intermediates 132 were in turn obtained by a palladium} \\
catalyzed cross-coupling between the 4-chloro-5-iodo-2,6-dimethylpyrimidine 128 and \\
\text{appropriate stannanes 130 or alkene 129, followed by nucleophilic displacement of the 4-chloro} \\
in pyrimidine 131 with sodium azide.}
\end{align*}
\]

Scheme 37. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from 2,6-diaminopyrimidin-4(3\textit{H})-one

\[
\begin{align*}
\text{Reagents and conditions: a) aq. ClCH}_2\text{CHO, DMA, r.t.; b) MeOH; c) PivCl, 90 \text{oC; d) POCl}_3, CH}_3\text{CN,} \\
\text{reflux; e) NIS, THF, r.t.; f) K}_2\text{CO}_3, \text{DMF, r.t.}
\end{align*}
\]
Shi et al.\textsuperscript{267} reported the synthesis started with 2,6-diaminopyrimidin-4-ol 134 (Scheme 37) in which treatment of 134 with aqueous chloroacetaldehyde provided the pyrrolo[2,3-d]pyrimidine 135 (42%). By performing this cyclization in the presence of \textit{N,N}-dimethylacetamide (DMA) as opposed to DMF, the desired product precipitated out as a 1:1 DMA complex, which facilitated its production on kilogram scale. The DMA was then easily removed by recrystallization from MeOH (95%). Protection of the amino group with PivCl gave amide 137 (93%). Deoxycyclonitration was best achieved with a complex mixture of products upon sequential treatment with POCl\textsubscript{3} and NIS. However, cleaving the pivaloyl group proved to be a difficult operation. After extensive experimentation, we found that the pivaloyl group could be efficiently removed if 138 was first alkylated with the commercially available chloromethylpyridine 140 to get 141 (80%).

\textbf{B.6 Name reactions}

The chemistry related to the present work will be reviewed and includes


B.6.2. Sonogashira coupling.

B.6.3. Ullmann coupling.

\textbf{B.6.1 Gewald reaction}

\textbf{Scheme 38.} A general model of the Gewald reaction.

\begin{center}
\begin{tikzpicture}
  \node (142) [draw, shape=rectangle] {$R_1\begin{array}{c}\text{O}\end{array}R_2$};
  \node (143) [draw, shape=rectangle, right of=142, xshift=2cm] {$S$};
  \node (144) [draw, shape=rectangle, right of=143, xshift=2cm] {$X=\text{COOC}_2\text{H}_5$};
  \node (145) [draw, shape=rectangle, right of=143, xshift=2cm] {$X=\text{CN}$};
  \node (146) [draw, shape=rectangle, right of=144, xshift=2cm] {$X=\text{COOC}_2\text{H}_5$};
  \node (147) [draw, shape=rectangle, right of=145, xshift=2cm] {$X=\text{CN}$};
  \node (148) [draw, shape=rectangle, right of=147, xshift=2cm] {$R_2$};
  \node (149) [draw, shape=rectangle, right of=148, xshift=2cm] {$R_1$};
  \node (150) [draw, shape=rectangle, right of=149, xshift=2cm] {$\text{H}_2\text{N}$};

  \draw[->] (142) -- (143);
  \draw[->] (143) -- (144);
  \draw[->] (143) -- (145);
  \draw[->] (144) -- (146);
  \draw[->] (145) -- (147);
  \draw[->] (147) -- (148);
  \draw[->] (148) -- (149);
  \draw[->] (149) -- (150);

  \node at (142) [below] {$142$};
  \node at (143) [below] {$143$};
  \node at (144) [below] {$144$};
  \node at (145) [below] {$145$};
  \node at (146) [below] {$146$};
  \node at (147) [below] {$147$};

  \node at (143) [left] {$a$};
  \node at (144) [right] {$\text{R}_2$};
  \node at (145) [right] {$\text{R}_1$};
  \node at (146) [right] {$\text{H}_2\text{N}$};

  \node at (142) [above] {$\text{R}_1\text{O}\text{R}_2$};
\end{tikzpicture}
\end{center}

Reagents and conditions: a) base
Gewald reaction (Scheme 38) is a multi-component condensation reaction between sulfur, an α-methylene carbonyl compound and an α-cyanoester in the presence of morpholine as catalyst to give 2-aminothiophenes. The Gewald reaction is the most convergent and well-established approach for the preparation of multiple substituted 2-aminothiophenes.

**Scheme 39.** The proposed mechanism of the Gewald reaction.

![Chemical structure](image)

Reagents and conditions: a) base; b) $\text{S}_8$, base

The reaction mechanism of the Gewald reaction has been recently elucidated as shown in Scheme 39, the first step is a Knoevenagel condensation between the α-methylene carbonyl compound 148 and an α-cyanoester 144 to produce intermediate 149. Through an unknown mechanism, intermediate 150 reacts with elemental sulfur to afford intermediate 151, which is further converted to 2-aminothiophenes 153 via cyclization and tautomerization.

New procedures of the Gewald reactions have been developed under solvent free conditions. In 2014, Shearouse *et al.* reported the solvent-free synthesis of 2-aminothiophenes.
(R = H, Br, NO₂, etc.) via Gewald reaction. A one-step one-pot method for conducting the Gewald synthesis in presence of base catalyst was discovered.

B.6.2 Sonogashira coupling

Scheme 40. A general model for Sonogashira cross-coupling.

Reagents and conditions: a) Pd(0) or Pd(II) (cat)/ligand, Cu(I)-salt (cat)/base/solvent

In 1975, Sonogashira et al.²⁷⁰ reported the synthesis of symmetrically substituted alkynes via a coupling reaction between acetylene gas and aryl iodides or vinyl bromides in the presence of catalytic amounts of Pd(PPh₃)Cl₂ and CuI under mild conditions. Thus, the copper-palladium catalyzed coupling of terminal alkynes with aryl and vinyl halides to give alkynes is called the Sonogashira cross-coupling (Scheme 40). Typically, two catalysts, a zerovalent palladium complex and a halide salt of copper(I), are necessary for the reaction. The reaction also requires basic medium to neutralize the hydrogen halide produced as the byproduct of this coupling reaction. The reactivity order of the aryl and vinyl halides is I ≈ OTf > Br >> Cl.
**Scheme 41.** Mechanism of Sonogashira cross-coupling. Modified from Negishi, E. and Anastasia, L.\(^{271}\)

![Scheme 41](image)

Sonogashira cross-coupling is believed to involve oxidative addition-reductive elimination pathway (Scheme 41), although the mechanism is not clearly understood.

**Scheme 42.** Synthesis of \(N-(7\text{-benzyl-4-methyl-5-(phenylethynyl)-7H\text{-pyrrolo}[2,3-\text{d]}\text{-pyrimidin-2-yl})-N\text{-pivaloylpivalamide 164.}\)

![Scheme 42](image)

Reagents and conditions: a) Phenylacetylene, \(\text{Et}_3\text{N}\), tetrakis(triphenylphosphine) palladium (0), \(\text{CuI}\), r.t., DCM
In 2007, Gangjee et al.\textsuperscript{272} reported the synthesis of \(N\)-(7-benzyl-4-methyl-5-(phenylethynyl)-7\(H\)-pyrrolo[2,3-\(d\)]pyrimidin-2-yl)-\(N\)-pivaloylpivalamide \textbf{164} (Scheme 42) from \(N\)-(7-benzyl-5-iodo-4-methyl-7\(H\)-pyrrolo[2,3-\(d\)]pyrimidin-2-yl)-\(N\) pivaloylpivalamide and phenylacetylene \textbf{163} via a Sonogashira cross-coupling in the presence of tetrakis(triphenylphosphine)palladium(0) and CuI as catalysts in dichloromethane.

\textbf{B.6.3 Ulman coupling}

\textbf{Scheme 43.} A general model for Ullmann coupling.

\[\begin{align*}
\text{X} = \text{Cl, Br, I} & \quad \text{Y} = \text{S, O, NH} \\
\text{R}_1 \quad \text{R}_2 & \quad \text{R}_1 \quad \text{R}_2 \\
\text{165} & \quad \text{166} & \quad \text{167}
\end{align*}\]

Reagents and conditions: a) Cu (I) or Cu (II) (cat), ligand/base/solvent

Ullmann-coupling is the copper-catalyzed nucleophilic aromatic substitution between various nucleophiles with aryl halides (Scheme 43).\textsuperscript{273} The Ullmann coupling involves the formation of a C-O, C-N and C-S bond by the reaction between an aryl halide with phenol, aniline and thiophenol. Typically, Ullmann coupling requires harsh reaction conditions including high temperatures (> 200 °C), strong bases, and long reaction times. In addition, the classical Ullmann reaction is limited to electron deficient aryl halides and can only affords moderate yields. The application of modern variants of the Ullmann reaction employing palladium and nickel have widened the substrate scope of the reaction and rendered reaction conditions more mild.\textsuperscript{274} Although the reaction mechanism of the Ullmann reaction has been extensively studied, the exact mechanistic pathway is unknown.\textsuperscript{345} According to radical scavenger experiments and electron spin
resonance, radical mechanisms have been ruled out. Although the exact nature (oxidation state) of the Cu-intermediate is not known, the reaction is proposed to involve the formation of an organocopper compound (RCuX), which reacts with the other aryl reactant in a nucleophilic aromatic substitution.

**Scheme 44. Synthesis of thioether 170**

Palomo *et al.*\textsuperscript{275} have reported the formation of a C-S bond via Ullmann coupling (Scheme 44) at lower temperature (80-110 °C) with high yield (80-94%).

**Scheme 45. Synthesis of thioether 173 under microwave assisted Ullmann coupling condition.**

Reagents and conditions: a) NiCl\textsubscript{2}PPh\textsubscript{3}, K\textsubscript{2}CO\textsubscript{3}, NMP, µW, 195 °C, 1-6h, 67-89%

Microwave assisted organic synthesis has been widely used in organic synthesis, resulting in faster and cleaner reactions. In 2010, Chen and coworkers\textsuperscript{276} reported Nickle catalyzed Ullmann coupling (Scheme 45).
III. STATEMENT OF THE PROBLEM

The present section deals with the design and molecular modeling studies of compounds in the following four areas:

C.1. Combination chemotherapy potential in single agents

1) 2, 4-substituted thieno[2,3-\textit{d}]pyrimidines
2) 2, 4, 5- and 6-substituted thieno[2,3-\textit{d}]pyrimidines
3) 4- and 7-substituted pyrrolo[3,2-\textit{d}]pyrimidines

C.2. Inhibition of tubulin

1) 2- and 4-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidine
2) 2, 4- and 5-substituted pyrazolo[4,3-\textit{d}]pyrimidines

C.3. Selective \textit{pj}DHFR inhibitors

1) 6-(arylthio)pyrido[2,3-\textit{d}]pyrimidine-2,4-diamines

C.4. Folate receptors (FRs) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one carbon (1C) metabolism inhibitors

1) \textit{N}1-substituted 5-amino-1,6-dihydro-\textit{7H}-pyrazolo[4,3-\textit{d}]pyrimidin-7-ones
2) \textit{N}2-substituted 5-amino- 2,6-dihydro-\textit{7H}-pyrazolo[4,3-\textit{d}]pyrimidin-7-ones
3) 6-substituted 2-amino-3,7-dihydro-\textit{4H}-pyrrolo[2,3-\textit{d}]pyrimidin-4-ones
C.1. Combination chemotherapy potential in single agents

Angiogenesis is the process for revascularization where new blood vessels form from pre-existing ones.\textsuperscript{277} Although angiogenesis is essential in physiological processes such as wound healing, it also participates in pathological conditions known as angiogenesis-dependent diseases. Angiogenesis is a hallmark of various diseases, including cancers, as tumors depend on a constant supply of oxygen and nutrients to grow.\textsuperscript{278} To grow beyond 2–3 mm in size, solid tumors depend on angiogenesis to meet their demand for nutrients, oxygen and proteolytic enzymes.\textsuperscript{279} It is an elaborate multistep process and is tightly regulated by a balance between proangiogenic and antiangiogenic factors.\textsuperscript{61} Hypoxia or low oxygen tension is the primary factor in the induction of angiogenesis where tumors secrete pro-angiogenic growth factors such as VEGF (Vascular endothelial growth factor), PDGF (Platelet-derived growth factor), and EGF (Epidermal growth factor).\textsuperscript{280} These growth factors bind to their respective receptor tyrosine kinases (RTKs) such as VEGFR, PDGFR-\(\beta\), and EGFR and stimulate the process of angiogenesis resulting in tumor growth, survival and metastases.\textsuperscript{279, 281} RTKs are the enzymes that catalyze the transfer of the \(\gamma\)-phosphate of ATP to tyrosine residues of protein substrates. RTK families are overexpressed in cancer cells.\textsuperscript{282}

Antiangiogenic agents (AA) have been identified as a relatively new treatment for cancer.\textsuperscript{278, 282, 283} Consequently, multi-RTK inhibition in cancer chemotherapy has emerged as a promising approach and its validity has been highlighted by the approval of several multi-RTK inhibitors including sorafenib (inhibits VEGFR-2, VEGFR-3, PDGFR-\(\beta\), and Raf kinase) and sunitinib (inhibits VEGFRs, PDGF\textsubscript{Rs} and c-kit).\textsuperscript{284} However, the antiangiogenic treatment only prevents blood supply to the tumor but does not destroy cancer cells. Additionally, RTK inhibitors that function by inhibition of a single RTK are prone to resistance by numerous mechanisms.
including redundant pathways, point mutations in the ATP-binding site and upregulation of additional RTKs.\textsuperscript{282, 283} Also, such single agents could afford synergic effects as anti-tubulin effects can exert their cytotoxic effect as soon as or even during transient tumor vasculature normalization caused by the antiangiogenic component.\textsuperscript{61} Additionally, the lack of long-term therapeutic efficacy is prevalent with the current RTK inhibitors.\textsuperscript{285, 286} Furthermore, antiangiogenic agents causing vascular regression could increase intra tumoral hypoxia.\textsuperscript{287} This might induce radio-resistance, chemo-resistance, and even anti-angiogenesis resistance since many pro-angiogenic molecules and factors contributing to tumor aggression are mainly released by tumor cells within hypoxic regions.\textsuperscript{288} Therefore, antiangiogenic agents need to be combined with radiotherapy and/or chemotherapy to provide effective treatment to achieve additive or synergistic effects in cancer treatment.\textsuperscript{289, 290}

Yoshizawa et al.\textsuperscript{291} have reported enhanced tumor penetration and \textit{in vivo} antitumor activity of cytotoxic paclitaxel in combination with the VEGFR2 inhibitor semaxanib in Colon-26 solid tumor-bearing mice. Tumors treatment with semaxanib contained significantly smaller hypoxic regions compared with the nontreated control group, suggesting that structural normalization of the tumor vasculature resulted in an improvement in tumor vessel functions, including oxygen supply. Immunostaining for endothelial cells and pericytes showed that the treatment with semaxanib enhanced the pericyte coverage of the tumor vasculature. Treatment with semaxanib increased the distribution of paclitaxel in the core region of the tumor, hence decreasing the ratio of their peripheral distribution. These results suggest that the structural and functional normalization of the tumor vasculature by the treatment with semaxanib to reach the deeper regions within tumor tissues leads to a more potent antitumor activity of paclitaxel. This strategy of utilizing the VEGFR2 inhibition based vascular normalization phase to enhance blood supply and
the delivery of cytotoxic agents may also address the problem of dose-limiting toxicities as there is decreased peripheral distribution of the cytotoxic agent.\textsuperscript{291}

The highly dynamic microtubules (MTs) play an essential role in mitosis and cell division.\textsuperscript{6} Microtubule targeting agents (MTAs) are highly effective drugs used for the treatment of solid tumors and hematological malignancies and one of the most significant drug classes for cancer chemotherapy.\textsuperscript{3, 6} Tubulin binding agents such as paclitaxel and Vinca alkaloids are widely used to treat solid tumors and hematological malignancies.\textsuperscript{6, 20} Antiangiogenic agents and cytotoxic agents in combination chemotherapy are more effective in cancer treatment than either agent alone.\textsuperscript{111, 289, 292} Single agents with dual antiangiogenic and cytotoxic activities significantly diminished tumor growth, tumor metastasis and angiogenesis and are superior to docetaxel and sunitinib in xenograft mice models, remarkably without any toxicity.\textsuperscript{293, 294} Such single agents with multiple mechanisms of action are commonly referred to as designed multiple ligands and show superior pharmacokinetic (PK), pharmacodynamic (PD) and safety profile compared to multiple drugs administered in combination.\textsuperscript{295, 296} These agents could potentially avoid drug-drug interactions associated with two or more agents.\textsuperscript{296-298} In addition, they could prevent or delay the emergence of resistance and toxicities.\textsuperscript{296, 298} Most significantly, single agents with dual cytotoxic and antiangiogenic activities simultaneously target proliferating tumor cells and the tumor vasculature. The molecule that acts simultaneously on multiple targets displays superior efficacy against advanced-stage diseases compared to compounds with high specificity for a single target.\textsuperscript{295} Thus, the cytotoxic component of these agents need not be as potent as conventional chemotherapeutic agents. Dosing of such an antiangiogenic agent with comparatively lower cytotoxic activity would mimic metronomic chemotherapy, which utilizes more frequent and low-dose administrations of cytotoxic agents compared with conventional chemotherapy.\textsuperscript{299} Several
clinical trials attest to the safety and efficacy of using antiangiogenic agents such as sunitinib and sorafenib with metronomic doses of cytotoxic agents.\textsuperscript{300-303} Such single agents with multi-targeting potential offer other advantages such as decreased cost and increased patient compliance\textsuperscript{297}, which can play a major part in the clinical success of therapy.

RTK-overexpressing endothelial cells are targeted by the antiangiogenic component of multi-targeted single agents targets and therefore, is typically targeted to tumor cells under normal circumstances.\textsuperscript{61} On the other hand, the cytotoxic component interferes with tumor cell division with less selectivity over rapidly dividing normal cells present in bone marrow, hair and cells lining the mouth and gut.\textsuperscript{304} Therefore, a fundamental challenge in developing single agents with a cytotoxic component is that the cytotoxic component should only eradicate tumor cells that are compromised via the antiangiogenic effect but should not cause toxicity to normal cells.\textsuperscript{111, 293} As a result, dose-limiting toxicities associated with cytotoxic agents could be avoided as the cytotoxic component of these single agents need not be as potent as conventional chemotherapeutic agents.

Decreased tumor growth, tumor metastasis and angiogenesis in xenograft mice models are observed with single agents with dual antiangiogenic and cytotoxic activities without any toxicity.\textsuperscript{111, 293, 294} The antiangiogenic effects of these compounds were due to inhibition of RTKs and the cytotoxic effects were due to inhibition of tubulin.\textsuperscript{111, 293} In these project studies, inhibition of VEGFR-2, PDGFR-\(\beta\) and EGFR were chosen as the targets for the antiangiogenic effects because of their crucial role in tumor angiogenesis. Moreover, the successful clinical and preclinical combinations of tubulin inhibitors like paclitaxel with antiangiogenic agents\textsuperscript{305} were also an essential factor in selecting tubulin as the potential cytotoxic targets.
C.1.1. Design of 2-, 4- substituted thieno[2,3-d]pyrimidines

Figure 36: Structure of compounds 174-181.

Table 4: Effects of 174-181 on cell proliferation, microtubule depolymerization and RTK inhibition.

<table>
<thead>
<tr>
<th></th>
<th>IC50 ± SD (MDA-MB-435) nM</th>
<th>EC50 (nM)</th>
<th>Kinase inhibition IC50 (nM)</th>
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<td>EGFR</td>
</tr>
<tr>
<td>IC50</td>
<td>EC50</td>
<td></td>
<td>VEGFR-2</td>
</tr>
<tr>
<td>174</td>
<td>96.6±5.3</td>
<td>1200</td>
<td>29.5 ± 3.1</td>
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<td>175</td>
<td>42.7±3.2</td>
<td>230</td>
<td>49.2 ± 5.1</td>
</tr>
<tr>
<td>176</td>
<td>7.0 ± 2.7</td>
<td>27</td>
<td>192.3± 6.2</td>
</tr>
<tr>
<td>177</td>
<td>3.4 ± 0.9</td>
<td>8</td>
<td>28.5 ± 3.9</td>
</tr>
<tr>
<td>178</td>
<td>183 ± 3.4</td>
<td>5800</td>
<td>-</td>
</tr>
<tr>
<td>179</td>
<td>&gt;10 µM</td>
<td>&gt;40 µM</td>
<td>-</td>
</tr>
<tr>
<td>180</td>
<td>198 ± 23</td>
<td>8000</td>
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</tr>
<tr>
<td>181</td>
<td>61.5 ± 5.6</td>
<td>807</td>
<td>-</td>
</tr>
<tr>
<td>CA4</td>
<td>4.4±0.46a</td>
<td>9.8a</td>
<td>-</td>
</tr>
<tr>
<td>Sunitiniba</td>
<td>-</td>
<td>-</td>
<td>172±19.4</td>
</tr>
<tr>
<td>Erlotiniba</td>
<td>-</td>
<td>-</td>
<td>1.2 ± 0.2a</td>
</tr>
</tbody>
</table>

a Results previously published.111 b ND: Not determined.
Gangjee et al.\textsuperscript{306} published pyrrolo[3,2-\textit{d}]pyrimidines \textbf{174-175} (Figure 36) that are watersoluble colchicine site microtubule depolymerizing agents with sub micromolar potency against cellular proliferation. Compound \textbf{174} showed 2-digit nanomolar (GI\textsubscript{50}) inhibitor of 8 tumor cell lines in the NCI 60 cell line.\textsuperscript{306} Compounds \textbf{174-175} exhibited VEGFR-2, PDGFR-\textit{b}, and EGFR kinase inhibitory activities (Table 4) and also displayed impressive activities against both Pgp- and \textit{\beta}III-tubulin-overexpressing cancer cell lines.\textsuperscript{293}

Gangjee and coworkers\textsuperscript{307, 308} also reported thieno[3,2-\textit{d}]pyrimidines \textbf{176-177} (Figure 36) as single agents acting as MTAs, along with inhibitors of VEGFR-2, PDGFR-\textit{b} and EGFR (Table 4). Compounds \textbf{176-177} were designed as isosteric replacement or scaffold hopping of compounds \textbf{174-175}. Compounds \textbf{176-177} showed remarkable activities against both Pgp- and \textit{\beta}III-tubulin-overexpressing cancer cell lines (Table 4). Compounds \textbf{176} and \textbf{177} with the thieno[3,2-\textit{d}]pyrimidine scaffold are 14- and 12-fold better in cell proliferation activity than the pyrrolo[3,2-\textit{d}]pyrimidine scaffold containing compounds \textbf{174} and \textbf{175}, respectively. There is significant 44- and 29-fold improvement of microtubule depolymerizing activity for compounds \textbf{176} and \textbf{177} compared to \textbf{174} and \textbf{175}, respectively. Compound \textbf{177} is 2-fold better than compound \textbf{175} in EGFR kinase inhibitory activity.

On the other hand, compounds \textbf{178-181} (Figure 36), were designed as MTA and RTK inhibitors to explore the SAR of the pyrrolo[2,3-\textit{d}]pyrimidine scaffold.\textsuperscript{56} Compound \textbf{178} was tested in the preclinical screening program of the National Cancer Institute in its 60-cancer-cell-line panel, and found to inhibit the proliferation of most of the cancer cell lines with a GI\textsubscript{50} in the nanomolar range.\textsuperscript{309, 309, 310} Compound \textbf{178} was discovered to be a colchicine site binding and also to overcome the two most clinically relevant tumor resistance mechanisms that limit the activity of microtubule targeting agents: overexpression of Pgp\textsuperscript{311, 312} and \textit{\beta}III-tubulin.\textsuperscript{313-317} Gangjee and
coworkers\textsuperscript{318} reported pyrrolo[2,3-\textit{d}]pyrimidines 180 where isosteric replacement of the oxygen atom of the 4’-OCH\textsubscript{3} of 178 with a sulfur moiety generated 180 to determine the nature of heteroatom substitution to hydrogen bond (HB) strength.\textsuperscript{319} Conformational analysis via molecular modeling and \textsuperscript{1}H NMR studies of 178 and 179 suggested that the methyl group at the 4-position of the aniline nitrogen of 178 restricted the free rotation of bond “\textit{a}” as well as bond “\textit{b}” (Figure 36) and thus restricts the conformation of the aniline ring in 179.\textsuperscript{320} Incorporation of tetrahydroquinoline rings as in 181 further restrict the “\textit{b}”- bond. Compound 181 showed 3- and 7-fold better potency compared to 178 in the antiproliferative effect in MDA-MB-435 cells and microtubule depolymerizing assays in A-10 cells, respectively (Table 4). Compound 180 displayed comparable potency to 178. Compound 179 was inactive towards both assays.\textsuperscript{56} Unfortunately, for compounds 178-181, the biological evaluation of several RTK-overexpressing cancer cell lines showed poor RTK inhibitory activities.\textsuperscript{318}

![Chemical structures](image_url)

\textbf{Figure 37:} Rationale for designing 182
Compounds containing the thieno[3,2-\(d\)]pyrimidine scaffold displayed extraordinary improvement in inhibition of cell proliferation and microtubule depolymerization activity compared to pyrrolo[3,2-\(d\)]pyrimidine. The kinase inhibitory activities were better (EGFR) or comparable. On the other hand, compounds with pyrrolo[2,3-\(d\)]pyrimidines were moderately active against cell proliferation and microtubule depolymerization compared to 174-177. These attributes prompted the design of thieno[2,3-\(d\)]pyrimidine derivatives 182-185 (Series I, Figure 37 and 38) as isosteres of the pyrrolo[2,3-\(d\)]pyrimidines 178-181 and as regioisomer of the thieno[3,2-\(d\)]pyrimidines 176-177 to exhibit inhibitory activity as MTAs and activities against several RTKs such as VEGFR2,\(^{321-323}\) PDGFR\(\beta\),\(^{323}\) EGFR.\(^{324}\) The thieno[2,3-\(d\)]pyrimidine is a promising scaffold with a broad spectrum of biological activities with diverging profiles of pharmacodynamics.\(^{325}\) Thienopyrimidine-based EGFR inhibitors are well known in the literature.\(^{325,326}\)
To better understand the binding of proposed compounds 182-185 in the colchicine binding site and the potential significance of thieno[2,3-\textit{d}]pyrimidine compounds, all the compounds 182-185 and the lead compound 178 were docked in Schrodinger Maestro\textsuperscript{156} using the X-ray crystal structure of tubulin (PDB code: 6BS2, 2.65 Å).\textsuperscript{327} Multiple low energy conformations were obtained from the docking analysis. As representative examples, Figure 39 shows the docked conformation of 178 (green) superimposed on the co-crystallized ligand, colchicine (pink). The pyrrolo[2,3-\textit{d}]pyrimidine scaffold of 178 forms hydrophobic interactions with Ala\textbeta{}314, Ala\textbeta{}352 and Ile\textbeta{}368 and occupies the region where the A ring of colchicine binds. The N1 of the pyrrolo[2,3-\textit{d}]pyrimidine makes a water-mediated hydrogen bond with the backbone of Cys\textbeta{}239. The \textsuperscript{N1}-CH\textsubscript{3} interacts with the Leu\textbeta{}253 and Leu\textbeta{}250 through hydrophobic interactions. The oxygen atom of the 4´-methoxyphenyl group lies within the pocket that consists of polar residues like Lys\textbeta{}350, Asn\textbeta{}256, and Thr\textbeta{}312. Additionally, the 2-methyl group creates hydrophobic interaction with Leu\textbeta{}240 and Leu\textbeta{}246. The docked score of 178 was -9.96 kcal/mol.
Figure 40: Superimposition of the docked pose of 178 (cyan) and 182 (pink) in tubulin (PDB ID: 6BS2). Compounds 182-185 displayed similar binding to 178 to the colchicine binding site of tubulin. Proposed compounds 182-185 showed docked scores that were better than 178 (-9.96 kcal/mol) in tubulin, suggesting an improvement in their binding compared to 178. The docked scores were in a range of -10.12 to -11.49 kcal/mol. As a representative example, Figure 40 shows the docked conformation of 182 (pink) superimposed with lead 178 (cyan). Compared to the pyrrole ring in the lead 178, the thiophene ring in the thieno[2,3-d]pyrimidine scaffold of 182 is much more amenable to the hydrophobic pocket of Alaβ352, Alaβ314, Alaβ315, Ileβ368 and Ileβ316. The rest of the molecule interacts similarly to the lead 178. The docked score for 182 is -11.49 kcal/mol which is better than the lead 178 (-9.96 kcal/mol)
Figure 41: Superimposition of the docked pose of gefitinib (green) and 182 (pink) in EGFR (PDB ID: 4WKQ).^{328}

Proposed compounds 182-185 were also docked in the X-ray crystal structure of EGFR co-crystallized with gefitinib (PDB ID: 4WKQ).^{328} Figure 41 displays docking analysis of 182 in the EGFR binding site as a representative of compounds from Series I. The thieno[2,3-d]pyrimidine scaffold of 182 forms hydrophobic interactions with Val726. The oxygen of 4′-methoxyphenyl group forms water-mediated hydrogen bond with Thr854. The phenyl ring makes hydrophobic interactions with Val845 and Leu844. The N-1 and N-3 make hydrogen bond with CSX-797. The mode of binding of 182 is similar to compounds 183-185, in series I. The docked scores of 182-185 are -9.15, -8.36, -9.02, -9.28 kcal/mol, respectively, which are better than the docked score of gefitinib (-8.76 kcal/mol).
Figure 42A: Superimposition of the docked pose of axitinib (green) and 182 (pink) in VEGFR-2 (PDB ID: 4AG8).\textsuperscript{329} 42B: Docked pose of 182 (pink) in the crystal structure of homology model PDGFR-β.\textsuperscript{294}

Figure 42A shows the docked conformation of 182 (pink) superimposed on the co-crystallized ligand, axitinib (green) in the crystal structure of VEGFR-2.\textsuperscript{329} The thieno[2,3-d]pyrimidine scaffold of 182 is stabilized by the hydrophobic interactions with Leu889, Val899 Val914 and Val916. The N3 of 182 makes hydrogen bond with the backbone of Cys1045. The N4-CH3 is oriented towards the hydrophobic pocket formed by the Leu1035 and Phe1047. The best-docked pose of 182 had docked score of -13.21 kcal/mol, which is better than the gefitinib docking score -12.02 kcal/mol. All of the proposed compounds in the Series I also displayed docked scores within 1 kcal/mol of -13.21 kcal/mol in VEGFR-2, indicating these compounds should have VEGFR-2 activity.

The docked conformations of 182 (pink) in our validated homology model\textsuperscript{294} of PDGFR-β indicated that the thieno[2,3-d]pyrimidine scaffold of 182 forms hydrophobic interactions with Ala713, Ala764 and Ile766 (Figure 42B). The 4′-methoxyphenyl group is oriented to the pocket
formed by Cys843 and Lys762. The thiophene ring in 182 is oriented to the hydrophobic pocket lined by Ile766. This suggests that a thieno[2,3-d]pyrimidine would lead to a considerable increase in the PDGFR-β activity. The best-docked poses of 182 had docked scores of \(-8.30\) kcal/mol. All the proposed compounds in the Series I also displayed docked scores within 1 kcal/mol of -8.30 kcal/mol in PDGFR-β, suggesting that these analogs would have good PDGFR-β inhibitory activity.

Figure 43: Cyclopenta[d]pyrimidines 186-187 and thieno[3,2-d]pyrimidines 176 and 188 with MTA and RTK activity

Table 5: Effects of 176, 186-188 on cell proliferation, microtubule depolymerization and RTK inhibition.

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50})±SD (MDA-MB-435) nM</th>
<th>EC(_{50})± SD (A-10 cells) nM</th>
<th>Kinase inhibition IC(_{50}) (nM)</th>
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<td></td>
<td>EGFR</td>
<td>VEGFR2</td>
<td>PDGFRβ</td>
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<td>186</td>
<td>7.0 ±0.7</td>
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<td>176</td>
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<td>Erlotinib(^a)</td>
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</table>

\(^a\): published data\(^{111}\)
Gangjee and coworkers\textsuperscript{319} also designed and evaluated the 5'-methoxynaphthalene cyclopenta[d]pyrimidine (187), variation of the 4'-methoxyphenyl cyclopenta[d]pyrimidine (186), which showed excellent MTA activities (Figure 43 and Table 5). Compound 187 displayed a ~3-fold improvement in antiproliferative effect, compared to 186 and was 2-fold better than the standard CA-4. Similarly, with the thieno[3,2-\(d\)]pyrimidine series 188 showed 4- and 3-fold improvement in antiproliferative effects than 176 and CA-4, respectively.\textsuperscript{308} Compound 188 is 9- and 3-fold better with regard to microtubule depolymerizing effects than 176 and CA-4, respectively. Additionally, 188 was tested for inhibition against EGFR, VEGFR-2 and PDGFR-\(\beta\) kinases that are overexpressed by tumor cells (Table 5). Compound 188 revealed 4- and 2-fold better activities in EGFR and PDGFR\(\beta\) kinase assays than 176.\textsuperscript{308} The improvements in activities of 187 and 188 compared to 186 and 176, could be attributed to their bulkier naphthyl group, which improves their binding in the colchicine binding site in tubulin and respective kinases.

\begin{figure}[h]
\centering
\includegraphics[width=0.3\textwidth]{compound_189.png}
\caption{Series II}
\end{figure}

Series II (Compound 189, Figure 44) with a thieno[2,3-\(d\)]pyrimidine ring was designed as bioisosteric analog of 187 and a regio analog of 188. The 5'-methoxy naphthalene group of 187-189 mimics the 4'-methoxyphenyl group of 182 (Figure 38). Proposed compound 189 displayed docked score (-12.04 kcal/mol) higher than compound 187 (-11.49 kcal/mol) and 188 (-11.66 kcal/mol) in tubulin, suggesting an improvement in their binding compared to 187 and 188.
Table 6: Antiproliferative and microtubule depolymerization activity of 190-195

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ ± SD in MDA-435 Cells (nM)</th>
<th>EC$_{50}$ for Microtubule Depolymerization in A·10 Cells (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>190</td>
<td>50.4 ± 2.4</td>
<td>691</td>
</tr>
<tr>
<td>191</td>
<td>16.7 ± 0.8</td>
<td>110</td>
</tr>
<tr>
<td>192</td>
<td>60.4</td>
<td>319</td>
</tr>
<tr>
<td>193</td>
<td>36.9</td>
<td>200</td>
</tr>
<tr>
<td>194</td>
<td>9.8 ± 0.9</td>
<td>21</td>
</tr>
<tr>
<td>195</td>
<td>3.8 ± 0.3</td>
<td>22</td>
</tr>
<tr>
<td>CA-4</td>
<td>4.4 ± 0.46</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Gangjee and coworkers$^{308,330}$ designed compounds 190-195 (Figure 45) as analogs of 186 and 176, respectively. Exploration of 2-position was attempted by removing the methyl group from 186 and 176 to afford 190 and 192, respectively. Compounds 191 and 193 were generated by incorporating an amino group at the 2-position to explore the importance of a 2-amino and hydrogen bond (if any) with corresponding amino acids at the colchicine site and RTK binding sites. Compounds 191 and 193 were 3- and 2-fold better than the corresponding 2-H compounds 190 and 192, respectively, in the antiproliferative assay (Table 6). Additionally, 191 and 193 were
6- and 1.5-fold better in potency regarding microtubule depolymerizing activity than 190 and 192, respectively. Conformationally restricted analogs of 190 and 192, by restricting the rotation around the “b” bond generated 194 and 195, respectively (Figure 45). Conformationally restricted analogs 194 and 195 were 5- and 16-fold better in antiproliferative assay in MDA-435 cells and 32- and 14-fold better in potency in microtubule depolymerization activity, respectively than 190 and 192.

![Chemical structures](image_url)

**Figure 46: Series III**

Compounds 196-197 were designed as bioisosteric analogs of 190-191 and regioisomers of 192-193 (Series III, Figure 46). Compounds 198-199, which are conformationally restricted analogs of 196-197, were designed by restricting the rotation of the “b” bond.

![Chemical structures](image_url)

**Figure 47.** N-(4′-methoxyphenyl)-N-methylquinazolin-4-amine 200-203 with MTA activity.
Table 7: Antiproliferative and microtubule depolymerization activity of 200-203

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ ± SD in MDA-435 Cells (nM)</th>
<th>EC₅₀ (nM) for Microtubule Depolymerization in A·10 Cells (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>CH₃</td>
<td>1.7 ± 0.1</td>
<td>2.12</td>
</tr>
<tr>
<td>201</td>
<td>Cl</td>
<td>0.6 ± 0.1</td>
<td>2</td>
</tr>
<tr>
<td>202</td>
<td>CH₃</td>
<td>1.1 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>203</td>
<td>Cl</td>
<td>0.7 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td>CA-4</td>
<td></td>
<td>4.4 ± 0.46</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Compound 200 (Figure 47) was reported as a potent inducer of apoptosis, binding at the colchicine site and an inhibitor of tubulin polymerization (EC₅₀ T47D=2 ± 0.1 nM). Compound 201 with a chloro substitution at the 2-position were synthesized by Gangjee and coworkers, demonstrated 3-fold better antiproliferative activities than 200 (Table 7). Restriction around the “b” bond on 200 and 201 generated compounds 202 and 203, respectively, which showed comparable antiproliferative activities toward MDA-435 tumor cells.

Table 8: In silico pKa and cLogP of 200-203

<table>
<thead>
<tr>
<th>R</th>
<th>pKa</th>
<th>cLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>CH₃</td>
<td>6.7</td>
</tr>
<tr>
<td>201</td>
<td>Cl</td>
<td>3.3</td>
</tr>
<tr>
<td>202</td>
<td>CH₃</td>
<td>6.66</td>
</tr>
<tr>
<td>203</td>
<td>Cl</td>
<td>3.22</td>
</tr>
</tbody>
</table>

cLogP and pKa were calculated using ACD labs pKa predictor.
Comparison of the pKa of protonated $N_1$ and cLogP for 2-H compounds (200, 202) and 2-Cl compounds (201, 203) suggests that 2-Cl substitution increases the cLogP and decreases the pKa (Table 8). The increased cLogP (increased lipophilicity) could increase the passive diffusion of the compounds. Additionally, decreased pKa will decrease ionization of the compound at physiological pH.\textsuperscript{334} Both of these effects could increase the intracellular concentration of the compounds with a 2-Cl, compared to the lead compounds with a 2-CH$_3$.

![Figure 48: Series IV](image)

Literature precedence shows bioisosteric replacement of the quinazolin-4(3$H$)-one scaffold by thieno[2,3-$d$]pyrimidin-4(3$H$)-one ring.\textsuperscript{325} Hence, bioisosteric replacement of the quinazoline core with a thieno[2,3-$d$]pyrimidine could be explored toward finding more active molecules for the development of anticancer agents. Thus, compounds 204-205 (Series IV, Figure 48) with 2-Cl derivatives of 2-CH$_3$ analogs 182 and 185 (Figure 38, series I) were designed. Compounds 204-205 are bioisosteric analogs of the quinazoline derivatives 201 and 203, respectively.
C.1.2. Design of 2-, 4-, 5- and 6-substituted thieno[2,3-d]pyrimidines

C.1.2.1 Design of 5-methylated thieno[2,3-d]pyrimidin-4-amine

Figure 49. Pyrrolo[3,2-d]pyrimidines 206-217 with MTA activity

Table 9: Antiproliferative and microtubule depolymerization activity of 206-217

<table>
<thead>
<tr>
<th>No.</th>
<th>IC$_{50}$ ± SD (nM)</th>
<th>EC$_{50}$ (nM)</th>
<th>No.</th>
<th>IC$_{50}$ ± SD (nM)</th>
<th>EC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td>96.6 ± 5.3</td>
<td>1200</td>
<td>213</td>
<td>ND</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>207</td>
<td>193 ± 39</td>
<td>5700</td>
<td>214</td>
<td>9.2 ± 1.8</td>
<td>78</td>
</tr>
<tr>
<td>208</td>
<td>18.3 ± 5.0</td>
<td>309</td>
<td>215</td>
<td>21.0 ± 3.6</td>
<td>39.2</td>
</tr>
<tr>
<td>209</td>
<td>4.3 ± 0.3</td>
<td>7.4</td>
<td>216</td>
<td>30.5 ± 0.8</td>
<td>76.5</td>
</tr>
<tr>
<td>210</td>
<td>13.4 ± 0.5</td>
<td>27.5</td>
<td>217</td>
<td>4.1 ± 0.1</td>
<td>3.31</td>
</tr>
<tr>
<td>211</td>
<td>1.3 ± 0.0</td>
<td>1.48</td>
<td>CA-4</td>
<td>4.4 ± 0.46</td>
<td>9.8</td>
</tr>
<tr>
<td>212</td>
<td>42.7 ± 3.2</td>
<td>233.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Gangjee and coworkers$^{335}$, published compounds 209-211 and 215-217 (Figure 49) as analogs of 206-208 and 212-214, respectively, which incorporate an additional CH$_3$ group at 5-position. There could be two effects of this 5-CH$_3$ group: (i) it could allow additional hydrophobic
interactions with the targets, and (ii) restricts the rotation around the N-phenyl bond (bonds “a” and “b”), thus providing conformational rigidity which could increase potency (Figure 49). Low energy conformations of the proposed compounds were generated using Schrödinger Maestro, and the number of conformations within 5 kcal/mol obtained for 206 and 209 were 14 and 9, respectively. Thus, 209, the N^5-CH₃ analog of 206, displays a lower number of conformations due to conformational restriction across bonds “a” and “b”. This 5-CH₃ moiety led to an increase in anti-proliferative activity by 22-,14- and 14-fold in 209, 210, and 211 compared to compounds 206, 207 and 208, respectively (Table 9). The microtubule depolymerization activities of 209, 210, and 211 were better than 206, 207, and 208 by 162-, 207-, and 208-fold, respectively. Compounds 212-214 and 215-217 were designed as conformationally restricted analogs of 206-208 and 209-211, respectively, by further restricting the “b” bond. These analogs also inhibited of EGFR, VEGFR-2, and PDGFR-β kinases. Thus, they could inhibit four distinct targets and were single agents with combination chemotherapy and multiple RTK inhibitory potential. These attributes make them ideal as leads for the design of inhibitory multitargeted agents- VEGFR-2, PDGFR-β, EGFR and microtubule assembly.

![Chemical structures](image)

**Figure 50: Series V**
Based on the improved activities of the 5-methylated analogs of pyrrolo[3,2-\(d\)]pyrimidines, the 5-methylated thieno[2,3-\(d\)]pyrimidines 218-220 and 221-223 (Series V, Figure 50) were designed using scaffold hopping with 209-211 and 215-217 (Figure 49), respectively. Compound 218 contains a 5-CH\(_3\) will restricts the rotation of both bonds “a” and “b” in compound 182 (Figure 50). Low energy conformations of 182 and the proposed compound 218 were generated using Schrödinger Maestro\(^{156}\) and the number of conformations within 5 kcal/mol obtained for the compounds are 12 and 9. Compounds 218-223 with a 5-CH\(_3\), displayed a lower number of conformations due to conformational restriction across bonds a and b. The design of compounds 221-223 has two purposes: (i) incorporation of methyl group at the 5-position in 185, 199 and 205; (ii) conformationally restricted analogs of 218-220, to improve potency.

C.1.2.2 Design of 5,6-dimethylated thieno[2,3-\(d\)]pyrimidin-4-amine

![Diagram of Compound 224](image)

**Figure 51:** Compound 224 with a thieno[2,3-\(d\)]pyrimidine scaffold

Shyyka *et al.*\(^{336}\) reported various thieno[2,3-\(d\)]pyrimidines, which showed remarkable anticancer activity. Compound 224 (Figure 51), 2-(benzylamino)-5,6-dimethylthieno[2,3-\(d\)]pyrimidin-4(3H)-one was found to be the most active compound among thieno[2,3-\(d\)]pyrimidine-4(3H)- ones which possessed cytotoxic activity on almost all cancer cell lines with mean growth inhibition of 51.01\%, where the most sensitive was the melanoma cell line MDA-MB-435 with GP (Growth Percent) -31.02\%. On the other hand, compound 182 (Figure 38) showed potent RTK and tubulin inhibition.\(^{307}\) The most commonly used strategy in the design of
multitargeted agents is the hybrid drug design which involves incorporating structural elements from different compounds that bind to their respective targets. If the pharmacophores for the respective targets overlap, the common structural features of the lead compounds can be "merged" resulting in a multitargeted single agent.

Figure 52: Hybrid structure of 225 from lead compounds 224 and 182.

Figure 53: Series VI

Compound 225 (Figure 52) was designed as a hybrid structure of 182 and 224 with RTK and tubulin inhibition that would afford an analog as a single agent with dual antiangiogenic and cytotoxic activities. Proposed compounds 226-229 (Series VI, Figure 53,) are the direct analog of 225. In compound 226, isosteric replacement of the 4'-OCH₃ with 4'-SCH₃ was done by
incorporating sulfur in place of oxygen at 4′-position. Compounds 227-228 were designed as conformationally flexible molecule of 225 and 226. Compounds 225 and 226 are designed by restricting bond “a” by incorporating a methyl group at the N4-position of 227 and 228. Successful binding to a protein requires a molecule to adopt a bioactive conformation. By limiting the number of conformations available to the unbound molecule, there is a lower entropic cost when the molecule binds. Conformational constrained molecules 225 and 226 will enhance the binding affinity to its intended target protein by reducing the conformational entropic costs upon binding compared to 227 and 228. Compound 229 was designed as a conformationally restricted analogs of 225 by further restricting the “b” bond by incorporating a 7-methoxy-3,4-dihydro-2H-benzo[b][1,4]oxazine to explore hydrogen bond acceptor (HBA) ability (if any) of the compound.

Figure 54A: Docked pose of 225 (pink) in colchicine binding site in tubulin (PDB ID: 6BS2). Figure 54B: Superimposition of docked poses of 225 (pink) and gefitinib (green) in EGFR crystal structure (PDB ID: 4WKQ).

Proposed compounds 225-229 were docked in the colchicine binding site in the X-ray crystal structure of tubulin (PDB code: 6BS2, 2.65 Å). Multiple low energy conformations were
obtained on docking. As representative examples, Figure 54A shows the docked conformation of 225 (pink) where the thieno[2,3-\textit{d}]pyrimidine scaffold of 225 forms hydrophobic interactions with Ile\textbeta{}316, and Ile\textbeta{}368. Additionally, the 5- and 6-methyl groups are positioned towards the hydrophobic residues Met\textbeta{}257, Ala\textbeta{}314, and Ala\textbeta{}352. The N1 of the thieno[2,3-\textit{d}]pyrimidine makes water-mediated hydrogen bond with Cys\textbeta{}239. The N\textsuperscript{4}-\textit{CH}_3 interacts with Leu\textbeta{}246, Ala\textbeta{}248 and Leu\textbeta{}253 through hydrophobic interactions. The oxygen atom of the 4′-methoxyphenyl group lies within the pocket that consists of polar residues like Lys\textbeta{}350, Asn\textbeta{}256, and Thr\textbeta{}312. Moreover, the 2-methyl group creates hydrophobic interactions with Leu\textbeta{}240, Ala\textbeta{}248 and Leu\textbeta{}250. The docked score of 225 was -12.22 kcal/mol, better than the lead 182 (-11.49 kcal/mol).

Docking analysis compounds 225-229 were done in the X-ray crystal structure of EGFR co-crystallized with gefitinib (PDB ID: 4WKQ).\textsuperscript{340} Figure 54B reveals the superimposed docking pose of 225 where the thieno[2,3-\textit{d}]pyrimidine scaffold of 225 forms hydrophobic interactions with Leu718, Val726 and Gly719. The oxygen of 4′-methoxyphenyl group forms water-mediated hydrogen bond with Thr854. The phenyl ring makes hydrophobic interaction with Leu844. The N-1 and N-3 make hydrogen bond with CSX-797. Compounds 225-229 have similar binding pose to 182. The docked scores of 225 is -9.20 kcal/mol, slightly better than its desmethyl analog 182 (-9.15 kcal/mol).

Figure 55A shows the docked conformation of 225 (pink) superimposed on the co-crystallized ligand, axitinib (green) in the crystal structure of VEGFR-2.\textsuperscript{329} The thieno[2,3-\textit{d}]pyrimidine scaffold of 225 is stabilized by hydrophobic interactions with Leu889, Val848, Val914, and Val916. The N-3 of 225 makes hydrogen bond interaction with the backbone of Cys1045. The N\textsuperscript{4}-\textit{CH}_3 is oriented towards the hydrophobic pocket formed by Leu1035 and
Phe1047. The best-docked pose of 225 had a docked score of -13.46 kcal/mol better than its desmethyl analog 182 (-13.21 kcal/mol).

**Figure 55A:** Superimposition of the docked poses of 225 (pink) and axitinib (green) VEGFR-2 (PDB ID: 4AG8)\(^\text{329}\) and 55B. Superimposition of the docked poses of 225 (pink) and axitinib (green) in the homology model of PDGFR-\(\beta\).\(^\text{294}\)

The docked conformations of 225 (pink) in our validated homology model\(^\text{294}\) of PDGFR-\(\beta\) indicated that the thieno[2,3-\(d\)]pyrimidine scaffold of 182 forms hydrophobic interactions with Ala713, Ala764 and Ile766 (Figure 55B). The 4’-methoxyphenyl group is oriented in the pocket formed by Cys843, Leu715 and Lys762. The thiophene ring with methyl at 5-and 6-position in 225 is oriented in the hydrophobic pocket lined by Ile766, Ala764, and Ala713. Thus, the idea of proposing methyl groups at 5- and 6-positions of thieno[2,3-\(d\)]pyrimidine would lead to a considerable increase in the PDGFR-\(\beta\) activity. The best-docked poses of 225 had a docked score of -8.38 kcal/mol, similar to its desmethyl analog (-8.30 kcal/mol). All the proposed compounds
in this series also displayed docked scores within 1 kcal/mol of -7.25 kcal/mol in PDGFR-β, suggesting that these analogs would have good PDGFR-β inhibitory activity.

C.1.3. Design of 4- and 7-substituted pyrrolo[3,2-\textit{d}]pyrimidines

![Chemical structures of compounds 206-207 and 230-231.]

Figure 56: Pyrrolo[3,2-\textit{d}]pyrimidines 206-207 and 230-231.

Table: 10: EGFR, VEGFR-2, PDGFR-\textit{β} kinase activities of compounds 206-207 and 230-231.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (nM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGFR</td>
</tr>
<tr>
<td><strong>206</strong></td>
<td>29.5±3.1</td>
</tr>
<tr>
<td><strong>230</strong></td>
<td>23.6±5.8</td>
</tr>
<tr>
<td><strong>231</strong></td>
<td>1.1 ±0.2</td>
</tr>
<tr>
<td><strong>207</strong></td>
<td>19.2±1.9</td>
</tr>
<tr>
<td><strong>CA-4</strong></td>
<td>-</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1±9.4</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2±0.2</td>
</tr>
</tbody>
</table>

Gangjee and coworkers\textsuperscript{293, 341} reported compounds 206-207 and 230-231 (Figure 56) containing the pyrrolo[3,2-\textit{d}]pyrimidine scaffold. Compounds 206-207 acted as MTAs\textsuperscript{293} and
showed RTK inhibition (Table 10). On the other hand, compounds **230-231** with a benzyl ring at the 7-position showed better activity in VEGFR-2 kinase (compounds **230-231** are 6-fold better than **206** in VEGFR-2) and comparable potency in EGFR and PDGFR-β. Compound **207** with no substitution at the 2-position were designed was designed to explore the effect of substituents in this position regarding antitubulin activity and showed 1.5-fold improvement in EGFR and VEGFR-2 activity compared to **206**.

![Structure](image)

**Figure 57:** Series VII

Compound **232** (Series VII, Figure 57) was synthesized towards the goal of identifying single agents with RTK and tubulin inhibitory activities in the same structure. Incorporation of a 2-H group on **230-231** scaffold would explore the effect of 7-benzyl substituted pyrrolo[3,2-\(d\)]pyrimidines without any substituents in the 2-position and would also evaluate the importance of the 2-CH\(_3\) and 2-NH\(_2\) substituents in **230** and **231**, respectively.

**C.2. Inhibition of tubulin**

Tubulin binding agents disrupt microtubule dynamics leading to mitotic arrest and cell death.\(^{342}\) Tubulin binding agents such as taxanes and vinca alkaloids are widely used for the treatment of solid tumors and hematological malignancies.\(^{343}\) However, the enormous clinical success of taxanes and vinca alkaloids has been compromised by two primary mechanisms of tumor resistance:
Overexpression of Pgp and the expression of the βIII-tubulin. Pgp overexpression is clinically observed in many tumor cell lines, particularly in patients who have received prior chemotherapy. Overexpression of Pgp resulted in poor response to taxol-based chemotherapy in patients with non-small cell lung cancer. The use of Pgp inhibitors in overcoming Pgp-mediated resistance was not successful due to intolerable side effects. Tubulin binding agents that are not substrates for Pgp (e.g. epothilones) represent a viable alternative strategy for circumventing Pgp-mediated resistance. Such agents would be extremely useful for patients that develop resistance due to Pgp overexpression.

The expression of βIII-tubulin is involved in clinical resistance to taxanes and vinca alkaloids in non-small cell lung, breast, ovarian, and gastric cancers. Colchicine-site binding agents were not susceptible to βIII-tubulin mediated resistance, which demonstrates the importance of developing anti-cancer drugs binding to the colchicine-site. Although there are no colchicine-site agents in the clinic, several agents, including CA4P (Fosbretabulin®) and CA1P (OXi4503) are currently in phase 1 and 2 clinical trials. The development of tubulin-binding agents that are less sensitive to Pgp and/or βIII-tubulin mediated resistance could result in a broader spectrum of antitumor activity and improved rates of survival.

C.2.1 Design of 2- and 4-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidine

![Figure 58: Compounds 233 with MTA activity](image-url)
Figure 59: Series VIII

Tricyclic 5,6,7,8-tetrahydrobenzofuro[2,3-\(d\)]pyrimidines with general structures 233 (Figure 58) have been reported by Gangjee and coworkers.\(^{349}\) Compound 233 displayed 23.3±1.9 and 61 nM inhibition in antiproliferative and microtubule depolymerization assays, respectively. It was of interest to identify the structural features of the tricyclic 5,6,7,8-tetrahydrobenzofuro[2,3-\(d\)]pyrimidines that contribute to antitubulin activity. Hence, isosteric replacement of 5,6,7,8-tetrahydrobenzofuro[2,3-\(d\)]pyrimidine in compound 233 was carried out to afford compound 234 (Series VIII, Figure 59) as a potential inhibitor of tubulin.

Figure 60. Cyclopenta[\(d\)]pyrimidines 186 and 235 with MTA activity

Gangjee and coworkers\(^{319}\) designed and evaluated the cyclopenta[\(d\)]pyrimidines 186 and 235, possessing significant MTA activities (Figure 60). It was noted that the replacement of a 4′-methoxyphenyl with 4′-methylthiophenyl lead to an improvement in the tubulin assembly inhibition by 3-fold.\(^{319}\)
Series IX (Compound 69, Figure 61) was designed as the 4′-thiomethylphenyl analog of 234. The methoxy group of the lead 234 is positioned near Thrα179; thus, 236 was designed in an attempt to study the effect of replacement of the oxygen in 234 with sulfur at the 4′-position. It has been widely known that acceptor (or donor) strengths correlate with affinity, leading to valuable insights into the SAR for given scaffolds. Different hydrogen bond acceptors (HBA) can help design molecules with better overall properties, as developing a high-affinity inhibitor involves finding a delicate balance between intermolecular interactions and the unfavorable desolvation penalty suffered when a ligand binds to an enzyme or receptor. The strength of HBA based on pKBHX database is as follows: dimethylsulfide (0.12) and ether (1.11). Based on this data, compound 236 is designed to engage Thrα179 in the tubulin colchicine binding site with a water-mediated hydrogen bond. Sulfides are considered as mild HBA and these compounds will also offer insights into the optimum HBA strength. The entropic penalty of sulfide is considerably less than that of an ether despite sulfide being a weak HBA, which can lead to an improved affinity for the sulfide over the ether. Proposed compound 236 displayed a docking score lower than -11.56 kcal/mol in tubulin, suggesting an improvement in its binding over that of 234 (-11.19 kcal/mol).
Gangjee and coworkers\textsuperscript{319, 354} also designed and evaluated \textbf{187} and \textbf{237} as microtubule targeting agents (Figure 62). Replacement of the 4′-methoxyaniline in \textbf{186} (Figure 60) with a 6-methoxytetrahydroquinoline (\textbf{237}, Figure 62) restricts the rotation of the unsaturated ring of quinoline across \textit{N}\textsuperscript{4}-phenyl bond by restricting the “b” bond. Compound \textbf{187} was designed by varying the 4′-methoxyphenyl in cyclopenta\textit{d}pyrimidines (\textbf{186}) to a 5-methoxynapthalene (\textbf{187}). Both compounds \textbf{187} and \textbf{237} showed excellent MTA activities.\textsuperscript{319} Compound \textbf{237} showed a 1.5-fold improvement in the antiproliferative effect compared to \textbf{186}. Compound \textbf{187} displayed a 3-fold improvement in antiproliferative effect, compared to \textbf{186} and was 2-fold better than the standard CA-4.\textsuperscript{319} This was attributed to the increased binding of the bulkier compounds \textbf{187} and \textbf{237} to the colchicine binding site in tubulin.

\textbf{Figure 63:} Series X
Series X (compounds 238-240, Figure 63) were designed as analogs of 234 (Figure 59). Restriction of the “b” bond of 234 were carried out following Gangjee and coworkers\textsuperscript{319} to generate compound 238. The 5′-methoxy naphthalene compound 239 mimics the 4′-methoxyphenyl group of the lead 234. The oxygen can interact with the Thr\textalpha 179 of tubulin through a water-mediated hydrogen bond. Compound 240 a N\textsuperscript{4}-H analog is proposed as more flexible analog of 239. Proposed compounds 238-240 displayed a docked score in a range of -10.56 to -11.68 kcal/mol in the tubulin-binding site of colchicine.

![Compounds 241-243](image.png)

**Figure 64.** Pyrimido[4,5-b]indole 241-243 with MTA activity

<table>
<thead>
<tr>
<th>Comp.</th>
<th>MDA-MB-435 IC\textsubscript{50} ± SD (nM)</th>
<th>EC\textsubscript{50} for microtubule depolymerization (nM)</th>
<th>Inhibition of tubulin assembly IC\textsubscript{50} ±SD (µM)</th>
<th>Inhibition of colchicine binding (% inhibition ± SD) at 5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>241</td>
<td>33.9 ± 3.4</td>
<td>130</td>
<td>1.2 ± 0.04</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>242</td>
<td>14.7 ± 1.5</td>
<td>130</td>
<td>1.4 ± 0.007</td>
<td>84 ± 0.5</td>
</tr>
<tr>
<td>243</td>
<td>130 ± 7.8</td>
<td>1200</td>
<td>2.3 ± 0.4</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>CA-4</td>
<td>4.4 ± 0.46</td>
<td>9.8</td>
<td>1.0 ± 0.09</td>
<td>99 ± 0.2</td>
</tr>
</tbody>
</table>

**Table: 11.** Antiproliferative and microtubule depolymerization activities of 241-243.
Gangjee and coworkers\textsuperscript{320} reported the tricyclic pyrimido[4,5-\textit{b}]indoles \textbf{241-243} (Figure 64), which vary the substitution at the 2-position. Compounds \textbf{241-243} were evaluated as tubulin inhibitors and as agents that can overcome Pgp and $\beta$III-tubulin mediated drug resistance. These compounds have radiolabeled colchicine displacement values between 62-84\% at 5 $\mu$M concentration (Table 11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure65}
\caption{Series XI}
\end{figure}

The combination of multiple aromatic rings has significant implications for “drug-like” properties, and simple, planar structures are often associated with poor physicochemical profiles and toxicity.\textsuperscript{355} Drug candidates show a higher success rate with one or more Sp3 hybridized carbon atoms than “flat” molecules, because of low aqueous solubility of purely aromatic compounds.\textsuperscript{356} One of the limitations of MTAs, particularly the taxoids, is their poor water solubility.\textsuperscript{357} Thus, water-soluble microtubule targeted agents are highly desired, and an enormous effort continues to chemically modify and/or formulate analogs of these agents to increase their water solubility. Increasing ‘aromatic proportion’ in a molecule has a detrimental effect on solubility.\textsuperscript{358,359} On the other hand, the fraction of Sp3 hybridized carbon atoms (Fsp3), in other words, the fraction of carbon atoms that are saturated correlates with water solubility.\textsuperscript{356} Based on this hypothesis, we replaced the unsaturated aromatic ring in the lead compounds \textbf{241-243} to design compounds \textbf{244-248} (Series XI, Figure 65) with the 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidine scaffold in an attempt to both probe the water
solubility as well as the ring interactions with the hydrophobic pocket. These compounds are direct analogs of 234 (Figure 59) with variations at the 2-position. It was of interest to observe the isosteric effect of replacement of the 2-CH$_3$ in 234, 236, and 238 (Figure 59, 61, 63) with 2-NH$_2$ to generate compounds 244, 245, and 246, respectively.

Figure 66A: Docked pose of 244 (magenta) in the colchicine binding site of tubulin. Figure 66B: Docked pose of 247 (cyan) in colchicine binding site of tubulin (PDB ID: 6BS2)$^{327}$

The 2-NH$_2$ group in the lead compound 242 and the designed compounds 244-246 is oriented towards the solvent and polar residues. As a representative example, in Figures 66A, in the docked pose of the 2-NH$_2$ of proposed compound 244 is oriented towards the solvent and polar residues Asp249 and Lys252. To further improve the potency, the 2-NH$_2$ in compound 244-246 was removed to generate compound 247-248 to explore the importance of a 2-NH$_2$ and hydrogen bond (if any) with corresponding amino acids at the colchicine site. These two compounds (247 and 248) will determine the importance of any polar group like NH$_2$ at the 2-position and/or any hydrophobic interactions at the colchicine site.

To better understand the binding of the proposed tricyclic ring compounds, proposed compounds 234, 236, 238-240 and 244-248 were docked in the colchicine binding site in the X-
ray crystal structure of tubulin (PDB ID: 6BS2, 2.65 Å). Docking resulted in multiple low energy conformations. Figure 66A and Figure 66B show the docked conformation of lead compound 244 (magenta) and 247 (cyan) in the colchicine site, respectively. The thieno[2,3-\textit{d}]pyrimidine portion of the scaffold of 244 and 247 form hydrophobic interactions with Ile\textbeta316, Leu\textbeta253, Ile\textbeta368 and Cys\textbeta239 of tubulin. The cyclohexene part of the scaffold creates hydrophobic interactions with Ala\textbeta352, Leu\textbeta246, Ala\textbeta314, and Ile\textbeta368. The \textit{N}1 of the thieno[2,3-\textit{d}]pyrimidine portion makes water-mediated hydrogen bond with Cys\textbeta239. The \textit{N}4-CH\textsubscript{3} interacts with Leu\textbeta253 through hydrophobic interactions. The oxygen atom of the 4’-methoxyphenyl group lies within the pocket that consists of polar residues like Lys\textbeta350 and Asn\textbeta256. The 2-NH\textsubscript{2} group resides in the pocket with polar residues Asp\textbeta249 and Lys\textbeta252. Docked scores of 244 and 247 were -12.23 and -12.24 kcal/mol, respectively. All the proposed compounds in this series also displayed a docked scores within 1 kcal/mol difference of -12.24 kcal/mol in tubulin, which is similar to the natural ligand colchicine (-13.28 kcal/mol) suggesting that these analogs would have good MTA inhibitory activity.

C.2.2 Design of 2-, 4- and 5-substituted pyrazolo[4,3-\textit{d}]pyrimidines

![Compounds 211, 217, 249-250 with MTA activity](image)

**Figure 67:** Compounds 211, 217, and 249-250 with MTA activity
Gangjee and coworkers\textsuperscript{353} reported compounds 211 and 217 (Figure 67) as a potent microtubule depolymerizing agent. They were discovered to be a colchicine-site binding agents and to overcome Pgp and βIII-tubulin mediated drug resistance clinically observed with paclitaxel and vinca alkaloids. Banerjee \textit{et al}.\textsuperscript{327} reported compounds 249-250 (Figure 67) where the purine and isoxazolo[5,4-\textit{d}]pyrimidine rings are bioisosteric replacement of the pyrrolo[3,2-\textit{d}]pyrimidine ring in compounds 211 and 217. Compounds 249 and 250 showed direct binding to the colchicine site in tubulin and significantly inhibited tumor growth in an A375 melanoma xenograft model. These compounds overcame clinically relevant multidrug resistance in a paclitaxel-resistant PC-3/TxR prostate cancer xenograft model. Furthermore, the 5-methyl group would also act to sterically restrict the number of conformations and render the possibility of getting bioactive conformation.\textsuperscript{335} Such conformational restriction has been reported in MTA drug design to provide conformation (s) more conducive to colchicine binding.\textsuperscript{320}

\textbf{Figure 68:} Series XII

Compounds 251-253 (Series XII, Figure 68) with a unique pyrazolo[4,3-\textit{d}]pyrimidine scaffold were designed as a bioisosteric replacement of all three rings (purine, isoxazolo[5,4-\textit{d}]pyrimidine, pyrrolo[3,2-\textit{d}]pyrimidine, Figure 67). As shown in Figure 68, the bioactive conformations of 251 and 253 are determined by three rotatable single bonds: 7-position C-N bond (bond \textit{a}), 1'-position C-N bond (bond \textit{b}), and 4'-position C-O bond (bond \textit{c}). Based on prior literature reports,\textsuperscript{320,335,346} conformational analysis via molecular modeling and \textsuperscript{1}H NMR studies
suggested that the methyl group on the $N^4$-aniline nitrogen restricted the free rotation of bond “a” as well as bond “b” and thus restricts the conformation of the anilino ring in 211 and 217. Successful binding to a protein requires a molecule to adopt a bioactive conformation. By limiting the number of conformations available to the unbound molecule, there is a lower entropic cost when the molecule binds. Therefore, investigation of conformationally restricted analog 251, where restriction of “a” bond was carried out by incorporating a $N^7$-CH$_3$ group on 252. Compound 253, the 1,2,3,4-tetrahydroquinoline derivative was designed from 252, by further restricting bond “b.”

Figure 69: Superimposition of the docked pose of 211 (cyan) and 251 (magenta) in the colchicine binding site in the X-ray crystal structure of tubulin (PDB ID: 6BS2, 2.65 Å).

Proposed compounds were docked in the colchicine binding site in the X-ray crystal structure of tubulin (PDB ID: 6BS2, 2.65 Å) to investigate the binding mode. Docking resulted in multiple low energy conformations. Figure 69 illustrates the docked conformation of lead compound 211 (cyan) and 251 (magenta) in the colchicine site. The pyrazolo[4,3-$d$]pyrimidine scaffold of 251 forms hydrophobic interactions with Ile$\beta$316, Ala$\beta$314, Ala$\beta$352 and Cys$\beta$239. The $N^1$-CH$_3$ of pyrazolo[4,3-$d$]pyrimidine interacts with Met$\beta$257 and Ala$\beta$314. Water mediated
hydrogen bond interaction with Cysβ239 was observed with the N4 of the pyrazolo[4,3- 
\textit{d}]pyrimidine. The N7-CH3 interacts with Leuβ253 and Leuβ246 through hydrophobic interactions.

The oxygen atom of the 4′-methoxyphenyl group lies within the pocket consisting of polar residues like Asnβ256, Thrβ312 and Asnβ348. The 5-Cl group resides in the pocket with hydrophobic residues Leuβ240 and Ala2β48. Docking score of 251 was -11.28 kcal/mol, which is better than the docking score of lead compound 211 (-10.95 kcal/mol).

\begin{figure}
\centering
\includegraphics[width=0.4\textwidth]{figure70}
\caption{Cyclopenta[\textit{d}]pyrimidines 186 and 254-256 with MTA activity}
\end{figure}

The concept homologation has been adapted and used in medicinal chemistry as an important strategy for molecular modification.\cite{Gangjee} Gangjee \textit{et al.}\cite{Gangjee} reported 186 and 254-256 (Figure 70) as potent microtubule targeting agents. Compounds 255 and 256 were designed by increasing the chain length at 4′-position by replacing 4′-OCH3 in 186 and 254 to 4′-OCH2CH3, respectively.

\begin{figure}
\centering
\includegraphics[width=0.4\textwidth]{figure71}
\caption{Series XIII}
\end{figure}
The homologation and branching of the 4′-OMe (251, Figure 64) to a 4′-OCH₂CH₃ (257) and 4′-OCH(CH₃)₂ (258) were designed to allow these groups to better penetrate the hydrophobic pocket in the colchicine site (Series XIII, Figure 71).

Figure 72: Pyrrolo[3,2-d]pyrimidines 211, 259-260 with MTA activity

Gangjee and coworkers reported 211 and 259-260 containing pyrrolo[3,2-d]pyrimidines with chlorine at 2-position (Figure 72). In compound 259 the 5′-methoxynapthalene variation of the 4′-methoxyphenyl (211) was designed, which showed 22.6- and 50 nM antiproliferative and microtubule depolymerization activity, respectively. A comparative activity was observed for the sulfur analog 260, which was designed with the sulfur as a bio-isosteric replacement of the oxygen on the phenyl ring on 211. Sulfides can mimic the HBA ability of the ether oxygen with decreased desolvation penalty.

Figure 73: Series XIV
Compounds 261-263 (Series XIV, Figure 73) were designed to explore the impact of electron density and hydrogen bond ability on the \(N^7\)-phenyl ring. Compound 261, the 5′-methoxynapthalene variation of the 4′-methoxyphenyl in the pyrazolo[4,3-\(d\)]pyrimidine 251 (Figure 68) was designed to evaluate the additional hydrophobic group and electronics effect on the \(N^7\)-substitution. The distance and the nature of heteroatom substitution affects the hydrogen bond (HB) strength.\(^{319}\) Thus, it was of interest to isosterically replace the oxygen atom of the 4′-\(OCH_3\) of 251 (Figure 68) with a sulfur moiety to design 262. Compound 263 with 3′-fluorine group was designed to evaluate the contribution of meta-electron-withdrawing moieties on biological activity. All the proposed compounds in this series also displayed docked scores within 1 kcal/mol of -9.38 kcal/mol in tubulin, suggesting that these analogs would have good MTA inhibitory activity.

C.3. Selective pjDHFR inhibitors

Pneumocystis organisms characterize a large group of species of atypical fungi with universal distribution, with specificity for a specific mammalian host.\(^{135}\) Targeting *Pneumocystis jirovecii* DHFR (pjDHFR) is one of the most efficient strategies to treat PCP infection.\(^{149}\) DHFR enzyme catalyzes the reduction of 7,8 dihydrofolate to the 5,6,7,8-tetrahydrofolate and inhibits with thymidylate biosynthesis and consequently DNA synthesis, as well as the inhibition of folate dependent formyl transferases causing inhibition of purine synthesis.\(^{126}\) Disruption in DNA, RNA and protein synthesis and death of the organism are caused by these inhibition. Pneumocystis infection is host-species specific infection. Most of the drugs were tested against *Pneumocystis carinii* DHFR (pcDHFR), which was presumed to be the causative species of PCP infection in humans.\(^{126, 153}\) *P. carinii* is responsible for human infections infecting rats and is different from
P. jirovecii. Development of the homology model for pjDHFR and isolation of pjDHFR, provided the amino-acid sequence differences between pjDHFR and pcDHFR, as well as human DHFR (hDHFR). The difference in amino acid sequence of the DHFR of P. carinii (pcDHFR) is 38% when compared to the DHFR of P. jirovecii (pjDHFR).\(^{113}\) The superimposition of the active site of pcDHFR and pjDHFR displays the amino acid differences present in the active sites of the two enzymes and emphasizes the challenges of designing and evaluating activity against the surrogate pcDHFR as inhibitors of pjDHFR. Therefore, drugs evaluated against the surrogate pcDHFR in-vitro may not translate into potent activity for the treatment of PCP infection in humans.\(^{134}\)

Isolated pjDHFR\(^{113}\) has been used to evaluate clinically used agents such as TMP (trimethoprim), PTX (piritrexim) and novel DHFR inhibitors.\(^{134,154}\) Selectivity ratio (IC\(_{50}\) hDHFR/IC\(_{50}\) pjDHFR) measurement of the agent for pjDHFR over hDHFR can be calculated from the inhibition of hDHFR compared with pjDHFR. Due to low selectivity for pjDHFR over hDHFR compounds, such as PTX and TMQ (trimetrexate) are much toxic, and this explains the reason for their discontinuation for the treatment of opportunistic infections. The selectivity of TMP for pjDHFR over hDHFR is 266-fold and is an aspect that contributes to its clinical success in PCP treatment. Potency for pjDHFR is highly desirable besides the selectivity. TMP is a poor inhibitor of pjDHFR and lacks in vivo efficacy as a single agent against parasitic infections and must be used with Sulfamethoxazole (SMX). A long-term goal of Gangjee and coworkers are to provide analogs with excellent potency along with high selectivity for pjDHFR. Such agents could be used alone as well as with sulfonamides and other drugs for the treatment of PCP infections in humans.

Due to the lack of an X-ray crystal structure of pjDHFR the design of pjDHFR inhibitors became very challenging. However, homology models can be used with refinement to model
pjDHFR in the absence of crystal structures.\textsuperscript{154, 155} Therefore, along with known hDHFR X-ray crystal structures,\textsuperscript{155} pjDHFR homology models can be used to design and develop potent and selective pjDHFR inhibitors. Another most important challenge in the discovery and development of inhibitors of pjDHFR is the inability to grow the organism outside the human lung. As a result, with the synthesized compounds it is difficult to develop a tissue culture for \textit{in vitro} studies or an animal model for \textit{in vivo} evaluation. Isolation and use of the pjDHFR enzyme is presently the only direct indicator that a compound could be effective (or ineffective) in the treatment of PCP infection in humans.

\textbf{Figure 74.} X-ray crystal of hDHFR published with pyrido[2,3-\textit{d}]pyrimidine \textit{264}.\textsuperscript{154}
Figure 75. Superimposition of active sites of hDHFR and pjDHFR. The pairs of amino acid residues shown are the residues that are different. The cyan ribbon and pink ribbon represent the active site and major amino acid difference in hDHFR (PDB: 4QJC, 1.62 Å)\textsuperscript{155} and pjDHFR (homology model)\textsuperscript{154}, respectively.

Table 12: Differences in amino acids between hDHFR and pjDHFR

<table>
<thead>
<tr>
<th>hDHFR</th>
<th>pjDHFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val 115</td>
<td>Ile 123</td>
</tr>
<tr>
<td>Ile 60</td>
<td>Leu 65</td>
</tr>
<tr>
<td>Ser 59</td>
<td>Ser 64</td>
</tr>
<tr>
<td>Phe 31</td>
<td>Met 33</td>
</tr>
</tbody>
</table>

The X-ray crystal of hDHFR is published with a pyrido[2,3-d]pyrimidine 264 (Figure 74).\textsuperscript{154, 155} The amino acids of hDHFR and the homology model sequence of pjDHFR were superimposed on the hDHFR X-ray crystal published with pyrido[2,3-d]pyrimidines in order to...
study the differences in the active site of hDHFR and the pjDHFR (Figure 75). The amino acids are quite distinct that constitute the active site of pjDHFR and hDHFR (Table 12). The hDHFR active site contains Phe31, Ile60, and Val115, whereas the active site of pjDHFR contains Met33, Leu65, and Ile123. Occupying the same location in the active sites, such as Met33 (in pjDHFR) vs. Phe31 (in hDHFR) and Ile 123 (in pjDHFR) vs. Val115 (in hDHFR), possess varied shapes, sizes and electronic properties. Such differences can be exploited for the design and development of inhibitors to provide potency and selectivity for pjDHFR over hDHFR.

C.3.1. Design of N°-substituted pyrido[2,3-d]pyrimidine-2,4-diamines

Table 13. Inhibition Concentrations (IC$_{50}$) against pjDHFR and hDHFR and selectivity ratios of pyrido[2,3-d]pyrimidines with NH-substitution at 6-position.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R</th>
<th>pjDHFR (nM)</th>
<th>hDHFR (nM)</th>
<th>Selectivity Ratios [hDHFR/pjDHFR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>265</td>
<td>H</td>
<td>300</td>
<td>190</td>
<td>1</td>
</tr>
<tr>
<td>266</td>
<td>4'-CH$_3$</td>
<td>620</td>
<td>2100</td>
<td>3</td>
</tr>
<tr>
<td>267</td>
<td>4'-OCH$_3$</td>
<td>400</td>
<td>3650</td>
<td>9</td>
</tr>
<tr>
<td>268</td>
<td>2',3'-(CH)$_4$</td>
<td>250</td>
<td>2100</td>
<td>8</td>
</tr>
<tr>
<td>269</td>
<td>3',4'-(CH)$_4$</td>
<td>400</td>
<td>2200</td>
<td>5</td>
</tr>
<tr>
<td>TMP</td>
<td></td>
<td>92</td>
<td>24500</td>
<td>266</td>
</tr>
<tr>
<td>PTX</td>
<td></td>
<td>41</td>
<td>2</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Gangjee et al.\textsuperscript{154} published 6-substituted pyrido[2,3-\(d\)]pyrimidine compounds 265-269 (Table 13). Isosteric replacement were carried out with NH at 6-position with the -CH\(_2\) moiety in PTX. Different electron-donating, electron-withdrawing and bulky substituents on the phenyl side chain were introduced to explore the SAR of this novel series of compounds. Compounds 265-269 displayed moderate potency for pjDHFR, but none of them were significantly selective for pjDHFR over hDHFR greater than 9-fold.

\textbf{Figure 76A.} Docked pose of 265 (purple) in the crystal structure of hDHFR (PDB: 4QJC, 1.62 Å, amino acids are depicted as light blue).\textsuperscript{154} \textbf{76B.} Docked pose of 265 (purple) in the homology model of pjDHFR (amino acids are depicted as light pink).\textsuperscript{154}

Docking analysis of 265-269 was performed in hDHFR and homology model of pjDHFR.\textsuperscript{154} In the X-ray crystal structure of hDHFR (Figure 76 A), the pyrido[2,3-\(d\)]pyrimidine scaffold of 265 stacked amid the Phe31, Phe34 and Leu22. The 2-NH\(_2\) makes ion-dipole interaction with Glu30 and water-mediated hydrogen bond with Thr136 and Phe134. The 4-NH\(_2\) makes a hydrogen bond with the backbone of Ile7. The N1-H of the pyrido[2,3-\(d\)]pyrimidine makes ion-dipole interaction with Glu30. The side chain phenyl ring makes hydrophobic interactions with Pro61, Ile60 and Leu67. In the homology model of pjDHFR (Figure 76 B), the
pyrido[2,3-\textit{d}]pyrimidine scaffold of 265 stacked between the Phe36, Leu25 and Ala12. The 2-NH\textsubscript{2} and N1-H make ion-dipole interaction with Thr144 and Asp32. The 4-NH\textsubscript{2} makes a hydrogen bond with the backbone of Val11. The side chain phenyl ring makes hydrophobic interactions with Pro66 and Leu65.\textsuperscript{361}

\textbf{Table 14.} Inhibition Concentrations (IC\textsubscript{50}) against pjDHFR and hDHFR and selectivity Ratios pyrido[2,3-\textit{d}]pyrimidines with N\textsuperscript{6}-CH\textsubscript{3} substitution.\textsuperscript{154,362}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Comp. & R & pjDHFR (nM) & hDHFR (nM) & Selectivity Ratios [hDHFR/pjDHFR] \\
\hline
270 & H & 2.2 & 57 & 26 \\
271 & 4ˊ-CH\textsubscript{3} & 2.4 & 32 & 13 \\
272 & 4ˊ-OCH\textsubscript{3} & 1.9 & 12 & 6.6 \\
264 & 2ˊ,3ˊ-(CH)\textsubscript{4} & 2.5 & 5.6 & 2.2 \\
\hline
TMP & & 92 & 24500 & 266 \\
PTX & & 41 & 2 & 0.05 \\
\hline
\end{tabular}
\end{table}
Gangjee et al.\textsuperscript{154} reported the 6-substituted pyrido[2,3-\textit{d}]pyrimidine compounds 264, 270-272 (Table 14). These compounds were designed by incorporating a \textit{N}\textsuperscript{6}-methyl group on 265-269. This methyl group was predicted to interact with Ile123 of pjDHFR and not with the shorter amino acid Val115 in hDHFR and thus confer both potency and selectivity for pjDHFR (Figure 77A and 77B) over hDHFR. Compounds 264, 270-272 (Table 10) displayed moderate potency and some had better selectivity for pjDHFR than compounds 265-269.

In hDHFR (Figure 74, 75), the corresponding Val115, being shorter by one carbon, may not interact with the \textit{N}\textsuperscript{6}-methyl group. This should improve selectivity as well as the potency of these compounds against pjDHFR over hDHFR. Further, the \textit{N}\textsuperscript{6}-methyl group restricts the number of possible conformations of the side chain phenyl group, therefore perhaps contributing to increased potency and selectivity.\textsuperscript{361} Compounds 264, 270-272 with the \textit{N}\textsuperscript{6}-methyl group are also more lipophilic than the corresponding \textit{N}\textsuperscript{6}–H analogs 265-269, thus, improving hydrophobicity and perhaps the cell penetration.\textsuperscript{361} Compounds 264, 270-272 with the \textit{N}\textsuperscript{6}-methyl group with
various electron-donating, electron-withdrawing, and bulky hydrophobic substituents were designed to explore the SAR and to afford potent and selective inhibitors for pjDHFR.\textsuperscript{154}

Table 15. Inhibition concentrations (IC\textsubscript{50}) against pjDHFR and hDHFR and Selectivity Ratios of pyrido[3,2-\textit{d}]pyrimidines with S-substitution at 6-position.\textsuperscript{353}

![Chemical structure](image_url)

<table>
<thead>
<tr>
<th>#</th>
<th>X</th>
<th>R</th>
<th>pjDHFR (nM)</th>
<th>hDHFR (nM)</th>
<th>Selectivity Ratios [hDHFR/pjDHFR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>273</td>
<td>NH</td>
<td>H</td>
<td>112</td>
<td>1526</td>
<td>13</td>
</tr>
<tr>
<td>274</td>
<td>NH</td>
<td>4´-OCH\textsubscript{3}</td>
<td>239</td>
<td>2459</td>
<td>10</td>
</tr>
<tr>
<td>275</td>
<td>NH</td>
<td>2´,3´-(CH)\textsubscript{4}</td>
<td>112</td>
<td>1098</td>
<td>10</td>
</tr>
<tr>
<td>276</td>
<td>NH</td>
<td>3´,4´-(CH)\textsubscript{4}</td>
<td>275</td>
<td>3185</td>
<td>12</td>
</tr>
<tr>
<td>277</td>
<td>S</td>
<td>3´-OCH\textsubscript{3}</td>
<td>0.96</td>
<td>450</td>
<td>469</td>
</tr>
<tr>
<td>278</td>
<td>S</td>
<td>2´,3´-(CH)\textsubscript{4}</td>
<td>0.031</td>
<td>0.526</td>
<td>17</td>
</tr>
<tr>
<td>279</td>
<td>S</td>
<td>3´,4´-(CH)\textsubscript{4}</td>
<td>0.061</td>
<td>4.12</td>
<td>67</td>
</tr>
<tr>
<td>280</td>
<td>S</td>
<td>4´-NO\textsubscript{2}</td>
<td>2.48</td>
<td>258</td>
<td>104</td>
</tr>
<tr>
<td>281</td>
<td>S</td>
<td>H</td>
<td>9.6</td>
<td>226</td>
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</tr>
<tr>
<td>TMP</td>
<td>-</td>
<td>-</td>
<td>92</td>
<td>24500</td>
<td>266</td>
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<tr>
<td>PTX</td>
<td>-</td>
<td>-</td>
<td>41</td>
<td>2</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Gangjee and coworkers\textsuperscript{353} reported pyrido[3,2-\textit{d}]pyrimidines with N\textsubscript{6}-H-substitution (273-276). Biological evaluation of 273-276 with NH linker at the 6-position of the pyrido[3,2-\textit{d}]pyrimidine scaffold displayed moderate potency and selectivity for pjDHFR (Table 15). A comparison of active sites of pjDHFR and hDHFR discloses several amino acid differences that could be exploited to gain potent and selective pjDHFR inhibitors (Figure 75). For instance, Met33/Phe31 in pjDHFR/hDHFR can affect binding due to their distinct steric and electronic properties. Isosteric replacement of the 6-position nitrogen with sulfur afforded 277-281 to further potentiate the activity for pjDHFR. Compounds 278 and 279, showed 3600 and 4500-fold better potency in pjDHFR, than their 6-NH analogs 275 and 276, respectively. Compounds 278 and 279 are 1.7 and 6-fold more selective than 275 and 276, respectively. Compared to the lead analog, sulfur linked analogs could change the bond angle, distance, and electronics of the side chain aryl group and cause a steric clash with Phe31 in hDHFR, whereas they appropriately fit with Met33 in pjDHFR and provide selectivity in pjDHFR over hDHFR.\textsuperscript{353}

\textbf{Table 16}. Bond angles and bond distance for C-X-C angle and C-X bond measured with Schrödinger Maestro.\textsuperscript{156}

<table>
<thead>
<tr>
<th>Entry</th>
<th>X</th>
<th>C-X Bond distance (Å)</th>
<th>C-X-C Bond angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>273</td>
<td>NH</td>
<td>1.41</td>
<td>132.8</td>
</tr>
<tr>
<td>281</td>
<td>S</td>
<td>1.76</td>
<td>100.6</td>
</tr>
</tbody>
</table>
Gangjee and coworkers\textsuperscript{353} reported bond angles and bond distances of the energy minimized conformations for representative analogs of 6-substituted pyrido[3,2-\textit{d}]pyrimidines 273 and 281 using Schrödinger Maestro.\textsuperscript{156} The increased C-S bond length and reduced C-S-C bond angle (Table 16) compared to the C-N bond length and C-N-C bond angle will increase the probability of a steric clash between the side chain aryl ring and the Phe31 in hDHFR (Figure 40). Compounds containing sulfur at the 6-position will thus decrease binding with hDHFR. On the other hand, the active site of pjDHFR has a flexible Met33 side chain (Figure 40) in place of Phe31 (hDHFR) and is anticipated to avoid the steric clash with the 6-substituted sulfur linked pyrido[3,2-\textit{d}]pyrimidines and improve selectivity in pjDHFR over hDHFR. Additionally, the S-linker at 6-position is expected to have less hydrophobic interaction with the shorter amino acid Val115 in hDHFR compared to Ile123 in pjDHFR and improves selectivity and potency in pjDHFR over hDHFR. This phenomenon was observed in compounds 277-281 in the docking study.

\textbf{Figure 78:} Series XV
Scaffold hopping has been widely applied by medicinal chemists to discover novel compounds. Compound 282 (Series XV, Figure 78) was designed as a hybrid of the 6-NH-substituted pyrido[2,3-\textit{d}]pyrimidine 265 and the 6-S-substituted pyrido[3,2-\textit{d}]pyrimidine 281. Isosteric replacement of NH- to S at the 6-position of the pyrido[2,3-\textit{d}]pyrimidine (Table 9, compounds 265-269) will generate the first compound 282 with a 6-S-substituted pyrido[2,3-\textit{d}]pyrimidine scaffold. Compound 282 can also represent a regioisomer of the 6-S-substituted pyrido[3,2-\textit{d}]pyrimidine 281.

![Figure 79: A. Docked pose of 282 (green) in the crystal structure of hDHFR (PDB: 4QJC, 1.62 Å). B. Docked pose of 282 (green) in the homology model of pjDHFR.](image)

To validate the hypothesis, we docked 282 in hDHFR (Figure 79 A) and the homology model of pjDHFR (Figure 79 B). In hDHFR the pyrido[2,3-\textit{d}]pyrimidine scaffold of 282 is stacked amid Phe31, Phe34, and Leu22. Ion-dipole interactions were observed between the 2-\textit{NH}_2 and N1- of the pyrido[2,3-\textit{d}]pyrimidine with Glu30. Additionally, water-mediated hydrogen bond with Thr136 and Phe134 was observed with the 2-\textit{NH}_2 of 282. The 4-\textit{NH}_2 makes a hydrogen bond with
the backbone of Ile7. The side chain phenyl ring makes hydrophobic interactions with Pro61, Ile60, and Leu67. The S-linker is expected to have less hydrophobic interaction with the shorter amino acid Val115 in hDHFR. The docked pose for 265 and 282 generated a docking score of -8.45 kcal/mol and -8.74 kcal/mol, respectively in hDHFR. In the homology model of pjDHFR, the pyrido[2,3-d]pyrimidine scaffold of 282 lies between the Phe36, Leu25, and Ala12. The 2-NH₂ and 4-NH₂ make ion-dipole interactions with Asp32. The 4-NH₂ makes a hydrogen bond with the backbone of Thr144. The side chain phenyl ring moves upwards and makes hydrophobic interactions with Phe36, Ile123, and Leu65. Sulfur is a bulkier linker compared to NH in the lead 265, and changes the angle compared to NH. The C-S-C angle of 282 and C-N-C angle of 265 were 100.5° and 131.1°, respectively (measured in Schrodinger Maestro). This decreased angle in 282 compared to 265 brings the side chain closer to Met33 in pjDHFR and produces a steric clash with Phe31 in hDHFR. Thus 282 is hypothesized to have greater selectivity for pjDHFR than hDHFR. The docked pose for 265 and 282 generated a docking score of -10.35 kcal/mol and -10.68 kcal/mol, respectively in pjDHFR.

![Diagram](image)

283 R = 3'-OCH₃  
284 R = 4'-OCH₃  
285 R = 3',4'-OCH₃  
286 R = 4'-F  
287 R = 4'-OCF₃

**Figure 80:** Series XVI
Compounds 283-287 (Series XVI, Figure 80) were designed as analogs of 282 with the pyrido[2,3-\(d\)]pyrimidine scaffold. Effects of electron-donating and electron-withdrawing groups are expected to determine the optimum electronics required for potency and selectivity for pjDHFR. Compounds 283-285 are anticipated to provide a structure-activity relationship through electron-donating groups (inductive and resonance). Compounds 286-287 were predicted to provide a structure-activity relationship through electron-withdrawing groups like fluorine and \(-\text{OCF}_3\) through inductive effects. All the proposed compounds displayed favorable docked scores for pjDHFR over hDHFR and were better than 265 and 281.

![Compounds 265 and 268](image)

**Figure 81:** Leads pyrido[2,3-\(d\)]pyrimidines.

Gangjee et al.\textsuperscript{154} reported pyrido[2,3-\(d\)]pyrimidines 265 (Figure 81).\textsuperscript{154} The potency of 265 against pjDHFR was IC\textsubscript{50} 300 nM and the selectivity over hDHFR was 1 (Table 13). To address the issue of selectivity, substituents with varying steric bulk and lipophilicity were probed at the 6-position. Compound 268 (Figure 81) was designed with a naphthyl group at the 6-position. Compound 268 showed better potency against pjDHFR (IC\textsubscript{50} 250 nM) and had the advantage of significantly enhanced selectivity over compound 265 (selectivity of 268 was 8). Due to the larger size of the naphthyl group in 268, the side chains of these proposed compounds were expected to sterically clash with the side chain Phe31 in hDHFR. This clash is avoided in pjDHFR, since the pjDHFR enzyme has a flexible Met33 residue instead of Phe31 (in hDHFR) at this position.
Compounds 288-289 (Series XVII, Figure 82) were designed as analogs of 282. Compound 288 is designed by an isosteric replacement of NH- of 268 to S-linked analog. Compound 289 is a regioisomer of 288, where the different orientations of the naphthyl groups on both 288 and 289 will suggest the different interactions with the amino acids in hDHFR and pjDHFR. The bulkier size of the naphthyl groups in 288-289 are predicted to sterically clash with the side chain Phe31, in hDHFR. This clash should be avoided as before in compounds 282-287 in pjDHFR, since the pjDHFR enzyme active site has a flexible Met33. Additionally, compounds 288-289 should evaluate the influence of electron-withdrawing groups on the side chain aryl group and its effect on potency and selectivity towards pjDHFR inhibition.

Figure 82: Series XVII

Figure 83: A. Docked pose of 289 (green) in the crystal structure of hDHFR (PDB: 4QJC, 1.62 Å). B. Docked pose of 289 (green) in the homology model of pjDHFR.
To validate the hypothesis compounds 288-289 were docked in hDHFR and the homology model of pjDHFR (Figure 83). In both the target, compounds 288-289 displayed similar interactions like 282 (Figure 79) and 268. The S-linker decreases the angle C-S-C in 288 compared to C-N-C in 268 and this creates a steric clash with Phe31 in hDHFR (Figure 83A), which is absent in pjDHFR (Figure 83B). The docked pose for 288 and 289 generated a docking score of -9.22, -9.15 kcal/mol and -10.86, -10.72 kcal/mol, respectively in hDHFR and pjDHFR. All of the proposed compounds in the Series XV-XVII displayed docked scores within 1 kcal/mol of -10.86 kcal/mol in pjDHFR, indicating these compounds should have excellent pjDHFR activity.

C.4. Folate receptors (FRs) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one carbon (1C) metabolism inhibitors

C.4.1. Design of N1-substituted 5-amino-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-ones

Proliferating tumor cells have unique metabolic requirements. These requirements are characterized by improved cell-autonomous nutrient uptake and a reorganization of metabolic pathways are needed for cell growth and division to support the biosynthesis of macromolecules. This includes the folate-dependent de novo synthesis of purine nucleotides and thymidylate. Folate receptors (FRs) mediate uptake of folates into cells by receptor-mediated endocytosis, whereas, the reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT) are facilitative folate transporters. FRα and FRβ, as well as PCFT are narrowly expressed in tissues where they perform specialized functions such as in the proximal tubules of the kidney where FRα contributes to reabsorption of folate from the urine. Notably, FRs in normal tissues are either
inaccessible to circulating folates (e.g., FRα in renal tubules) or are nonfunctional (FRβ in thymus). The PCFT is required for intestinal absorption of folates and folate transport from blood to cerebrospinal fluid across the choroid plexus. PCFT is expressed in the upper gastrointestinal tract where it functions as the major transporter for dietary folates at acidic pH. While PCFT is expressed in a number of other normal tissues (e.g., liver, kidney), it shows maximum activity at an acidic pH (pH < 7, optimum at pH 5–5.5), thus limiting PCFT mediated folate transport in most normal tissues. Several solid tumors (e.g., ovarian, non-small cell lung cancer) express PCFT, often together with FRα. In tumors, FRα is accessible to the circulation. Thus selective tumor targeting via PCFT and/or FRs is an attractive approach for development of antitumor antifolates. PCFT is active at the acidic pH typically associated with the tumor microenvironment, which provides additional tumor selectivity. FRβ is expressed in hematologic malignancies such as acute myeloid leukemia and in white blood cells of the myeloid lineage including tumor-associated macrophages (TAMs). Based on the different patterns of expression and functions of FRs, RFC, and PCFT in tumors and normal tissues, it is possible to design agents with tumor selective targeting. Folic acid-vindesine conjugate (vintafolide) was internalized by FRs and its cleavage intracellularly releases the cytotoxic vinca alkaloid. Another folic acid-tubulysin conjugate was in phase I clinical trials. In addition small molecule N-[4-[2propyn-1-yl][(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3Hcyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid (ONX0801) has substrate selectivity for RFs over RFC and has TS as its intracellular target. The principal aim is to design agents with transporter specificity and cytotoxic abilities, which does not require an intracellular cleavage mechanism to release the cytotoxic component as in to the conjugates.
previously developed. Premature cleavage (before reaching the tumor) of the cytotoxic vinca alkaloids or tubulysin results in unacceptable toxicities and consequent failure in clinical trials.

Cancer cells become dependent on one-carbon metabolism to support purine and thymidylate synthesis to sustain their proliferation. Reprogramming of energy metabolism is a hallmark in cancer development. Serine hydroxymethyltransferase (SHMT) catalyzes the reversible conversion of L-serine and tetrahydrofolate to glycine and 5,10-methylenetetrahydrofolate. This enzyme plays a pivotal role in one-carbon metabolism. It is involved in tumor cell metabolic reprogramming and is a recognized target of chemotherapy intervention. Folate-dependent one-carbon (C1) metabolism is situated in the mitochondria and cytosol and is a source of critical metabolites for proliferating tumors. Mitochondrial C1 metabolism, including serine hydroxymethyltransferase 2 (SHMT2), generates glycine for de novo purine nucleotide and glutathione biosynthesis. It is an important source of NADPH, ATP, and formate, which affords C1 units as 10-formyl-tetrahydrofolate and 5,10-methylenetetrahydrofolate for nucleotide biosynthesis in the cytosol. In cancer cells, serine is the major source of one-carbon units. In mitochondria, serine catabolic enzymes including SHMT2, 5, 10-methylene THF dehydrogenase 2 (MTHFD2) and 10-formyl-THF synthetase (reverse) (MTHFD1L) generate glycine and one-carbon units (i.e. formate) to sustain one carbon dependent nucleotide and amino acid biosynthesis in cytosol. The resynthesis of 10-formyl THF from formate in the cytosol is carried out by the trifunctional enzyme MTHFD1. The 10-formyl THF is utilized for purine nucleotide biosynthesis and can be further converted by MTHFD1 to 5,10-methylene THF for TS and SHMT1.

Comparison of mRNA profiles of 1,454 metabolic enzymes across 1,981 tumors spanning 19 cancer types identified SHMT2 and MTHFD2 as the most consistently overexpressed
enzymes. Metabolomic analysis of 219 extracellular metabolites from the NCI-60 cancer cell lines showed that glycine metabolism strongly correlated with cancer cell proliferation. Thus, mitochondrial one-carbon metabolism has been implicated as critical for the malignant phenotype. These findings, combined with a functional shortage of amino acids (e.g., glycine) in tumors, suggested a therapeutic opportunity for SHMT2 targeting in cancer.

Table 17. IC$_{50}$ Values (nM) for 5-Substituted Pyrrolo[2,3-$d$]pyrimidine Classical Antifolates in RFC-, PCFT-, and FR-Expressing CHO Cell Lines. Growth inhibition assays: IC$_{50}$ Values (nM) for 5-Substituted Pyrrolo[2,3-$d$]pyrimidines in KB human tumor sublines (expressing RFC, FR$\alpha$, and PCFT) and protection study results by added metabolites.

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFR$\alpha$</th>
<th>hPCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>R2</td>
<td>RT16</td>
</tr>
<tr>
<td>PMX</td>
<td>12 ± 1.1</td>
<td>216±8.7</td>
<td>114 ± 31</td>
</tr>
<tr>
<td>290</td>
<td>68.8 ± 21.2</td>
<td>&gt;1000</td>
<td>72.0±27.1</td>
</tr>
<tr>
<td>291</td>
<td>56.6 ± 5.8</td>
<td>&gt;1000</td>
<td>8.6 ± 2.1</td>
</tr>
<tr>
<td>292</td>
<td>196.4 ± 55</td>
<td>&gt;1000</td>
<td>33.5 ± 2.5</td>
</tr>
<tr>
<td>293</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
Gangjee and coworkers\textsuperscript{221} synthesized 5-substituted pyrrolo[2,3-\textit{d}] pyrimidine agents \textbf{290-293}, as dual-acting inhibitors of glycinamide ribonucleotide formyltransferase (GARFTase) and 5-aminomidazole-4-carboxamide ribonucleotide formyltransferase (AICARFTase) in \textit{de novo} purine nucleotide biosynthesis (Table 17). These compounds were substrates for FRs, PCFT, and RFC. Despite the moderate transporter activity via FRs and PCFT, \textbf{290-292} were not tumor cell specific. \textbf{PMX} (Table 17) has significant uptake through RFC, which is its major limitations with respect to a dose limiting toxicity.

\textbf{Table 18.} IC\textsubscript{50} Values (nM) for 5-Substituted Pyrrolo[3,2-\textit{d}]pyrimidine in RFC-, PCFT-, and FR-Expressing Cell Lines and KB human tumor sublines (expressing RFC, FR\textalpha, and PCFT).

<table>
<thead>
<tr>
<th>R2</th>
<th>RFC</th>
<th>FR\textalpha</th>
<th>FR\textbeta</th>
<th>PCFT</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>RT16</td>
<td>D4</td>
<td>R2/hPCFT4</td>
<td></td>
</tr>
<tr>
<td>294</td>
<td>&gt;1000</td>
<td>43</td>
<td>50</td>
<td>1.59</td>
<td>25.2</td>
</tr>
<tr>
<td>295</td>
<td>&gt;1000</td>
<td>516</td>
<td>2.13</td>
<td>1.29</td>
<td>309</td>
</tr>
<tr>
<td>296</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>26.6</td>
<td>6.3</td>
<td>327</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60(8)</td>
<td>13.2(2.4)</td>
</tr>
</tbody>
</table>
Table 19. IC₅₀ values for cell-free enzyme inhibition by pyrrolo[3,2-d]pyrimidine inhibitors. Results are shown as mean IC₅₀ values (± standard deviations), corresponding to the concentrations that inhibit growth by 50%, from at least four biological replicates. IC₅₀ values represent the interpolated concentrations of drug at which growth of 50% of cells was inhibited relative to vehicle-treated control cells. ND denotes “not determined.”

<table>
<thead>
<tr>
<th>In vitro enzyme assays [Kᵢ (µM)]</th>
<th>GARFTase</th>
<th>AICARFTase</th>
<th>SHMT2</th>
<th>SHMT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>294</td>
<td>ND</td>
<td>13.31 (3.16)</td>
<td>0.63 (0.29)</td>
<td>0.90 (0.06)</td>
</tr>
<tr>
<td>PMX</td>
<td>5.19 (1.63)</td>
<td>0.88 (0.56)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 20. IC₅₀ values for anti-proliferative activity toward engineered CHO and human tumor cell lines, and cell-free enzyme inhibition by pyrrolo[3,2-d]pyrimidine inhibitors. Proliferation inhibition assays were performed using human tumor cell lines, including HCT116 (colon cancer), H460 (lung cancer), and MIA PaCa-2 (pancreatic cancer). Results are shown as mean IC₅₀ values (± standard deviations), corresponding to the concentrations that inhibit growth by 50%, from at least four biological replicates. IC₅₀ values represent the interpolated concentrations of drug at which growth of 50% of cells was inhibited relative to vehicle-treated control cells.

<table>
<thead>
<tr>
<th>In vitro enzyme assays [Kᵢ (nM)]</th>
<th>HCT116</th>
<th>HCT116 SHMT2 KO</th>
<th>H460</th>
<th>MIA PaCa-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>294</td>
<td>2266(450)</td>
<td>108(66)</td>
<td>461(163)</td>
<td>3664(721)</td>
</tr>
<tr>
<td>PMX</td>
<td>293(81)</td>
<td>333(40)</td>
<td>165(59)</td>
<td>281(23)</td>
</tr>
</tbody>
</table>
Dual inhibition in the purine synthesis pathway is beneficial as the tumor cell cannot survive under conditions where resistance occurs developed to one of the targets. Past efforts on discovery and development of dual inhibition in the purine synthesis pathway have focused on developing agents to achieve: (i) transporter-specificity; (ii) dual inhibition of GARFTase and AICARFTase; and (iii) increased KB tumor cell inhibition. To investigate the contribution of the 7-NH moiety in 290-293 on the uptake and intracellular target inhibition, it was of interest to design compounds based on a novel scaffold. Gangjee and coworkers\textsuperscript{229,230} have recently reported 5-substituted pyrrolo[3,2-\textit{d}]pyrimidines 294-296 (Table 18-20). These are regio analogs of the 5-substituted pyrrolo[2,3-\textit{d}]pyrimidines 290-292. The proposed compounds evaluated the importance of a hydrogen bond donor at the 7-position in compounds 290-293. Compounds 294-296 inhibit cytosolic purine biosynthetic enzymes, GARFTase and/or AICARFTase, and C1 metabolism at SHMT1 and SHMT2 (Table 19).\textsuperscript{230} In vitro antitumor efficacy was established with the lead compound 294 toward lung, colon, and pancreatic cancer cells (Table 20).\textsuperscript{229,230} These discoveries established novel drug prototypes and structure-activity relationships for further development of multitargeted antitumor agents.

\textbf{C.4.1.1 Rationale for the design of pyrazolo[4,3-\textit{d}]pyrimidines}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure84.png}
\caption{A, B, C: Representation of the general structure of pyrroles, pyrazoles and pyridines; D: Representation of pyrazole structure, emphasizing the properties of the ring nitrogen atoms.}
\end{figure}
The pyrazolo[4,3-\(d\)]pyrimidine scaffold, an isostere of adenine, is an important drug-like scaffold, and its derivatives have shown a myriad of therapeutic applications including anti-inflammatory, anticancer, and anti-infectious effects.\(^{249,385}\) The pyrazole (Figure 84B) is a five-membered aromatic ring structure containing two vicinal nitrogen atoms. It has an acidic pyrrole-like nitrogen (Figure 84A) with a lone pair of electrons involved in aromaticity and a basic sp\(^2\)-hybridized pyridine-like nitrogen (Figure 84C), and three carbon atoms. Given the nature of the nitrogen, \(N\)-unsubstituted pyrazoles demonstrate amphoteric properties, acting as both acids and bases.\(^{386}\) While the acidic pyrrole-like NH group donates its proton, the basic pyridine-like nitrogen has the ability to accept protons even more readily. Nevertheless, substitutions on the ring can modulate these properties.\(^{386}\) Pyrazolo[4,3-\(d\)]pyrimidine, which is an isostere of purines, has acquired considerable importance due to its diverse, facile and general synthetic methodologies with great medicinal importance.\(^{249}\) Rosowsky \textit{et al.}\(^{387}\) reported (4-(2-(2-amino-6-oxo-6,9-dihydro-\(1H\)-purin-8-yl)ethyl)benzoyl)-\(L\)-glutamic acid analog with purine scaffold with potent biological activity as antifolates. Compounds 290-292 with 5-substituted pyrrolo[2,3-\(d\)]pyrimidine and 294-296 with 5-substituted pyrrolo[3,2-\(d\)]pyrimidine are excellent lead analogs for further structure optimization for improvement in multitargeted inhibition of GARFTase, and/or AICARFTase, and/or SHMT; along with selective transport by FRs and/or PCFT over RFC.

\[\text{Figure 85: Series XVIII}\]
Compounds 297-298 (Series XVIII, Figure 85) with a pyrazolo[4,3-d]pyrimidine scaffold was designed to probe the isosteric replacement of C-2 (Figure 84A) in the pyrrole ring to N-2 (Figure 84 B) in the pyrazole to determine the importance of the electronics in the five-membered ring. In compound 297-298, the acidic pyrrole-like N1-H group is alkylated, and the basic pyridine-like nitrogen N-2 has the ability to act as hydrogen bond acceptor (HBA) (Figure 84D, 85). The proposed compound 297-298 will evaluate the importance of HBA at the N-2 position and additional nitrogen atom at the 2-position in 297-298 will help to understand the role of the fused pyrazolo[4,3-d]pyrimidine ring system in anti-folate drug design.

To understand the binding of these compounds in transporters and intracellular targets, molecular modeling studies of 290-298 were carried out in the crystal structures for FRs, GARFTase, AICARFTase, SHMT2 using Schrödinger Maestro 2020.

Figure 86. Molecular modeling with human FRα (PDB ID: 5IZQ): Superimposition of docked poses of 295 (magenta) and 298 (green).
The docked scores of the proposed compounds in all the four targets (FRα, FRβ, GARFTase, AICARFTase, and SHMT2) were similar to that obtained for their respective lead compounds (±1.2 kcal/mol).

Figure 86 depicts the binding mode of 295 and 298 in the active site of FRα. For both compounds, the 2-NH$_2$ of 295 and 5-NH$_2$ of 298 form a hydrogen bond with Asp81. The 4-oxo of 295 and 7-oxo of 298 interacts with the side chains of Arg103 and Arg106 via hydrogen bond. The scaffolds are stabilized by a π–π stacking interaction with Tyr85 and Trp171. The acidic groups are in the proximity of hydrophilic amino acid residues. The α-COOH forms an ion-dipole interaction with Trp140 and the γ-COOH forms salt bridge with Lys136, and ion-dipole interactions with Trp102 and Gln100. The docked scores for 295 and 298 in FRα were -15.11 and -15.22 kcal/mol, respectively. This suggests that 298 would display a FRα transport similar to 295.

![Figure 86. Molecular modeling with human FRβ (PDB ID: 4KN2).](image)

**Figure 87.** Molecular modeling with human FRβ (PDB ID: 4KN2). Superimposition of docked poses of 295 (magenta) and 298 (green).
Figure 87 demonstrates the molecular modeling of 295 (magenta) and 298 (green) in FRβ. The scaffold is stabilized by π–π stacking interactions with Trp187 and Tyr101. The 2-NH$_2$ of 295 and 5-NH$_2$ of 298 interact via ionic bonds with Asp97. The 4-oxo group of 295 and 7-oxo of 298 interacts via a hydrogen bond with the Arg119 side chain. The 3-NH of 295 and 6-NH of 298 make a water mediated hydrogen bond with Arg122. The α-COOH forms an ion-dipole interaction with Trp156 and the γ-COOH forms ionic interaction with backbone of Arg152, and ion-dipole interaction with Trp118. The docked scores for 295 and 298 in FRβ were -15.24 and -15.38 kcal/mol, respectively. This suggests that 295 would display similar affinity as 298 in transport via FRβ.

![Molecular Modeling](image)

**Figure 88.** A. Molecular modeling of 295 (magenta) with human GARFTase (PDB ID: 4ZZI)  
88. B. Molecular modeling of 298 (green) with human GARFTase (PDB ID: 4ZZI)

Figure 88 show the molecular modeling of 295 (magenta) and 298 (green) in GARFTase. The 4-oxo of 295 and 7-oxo of 298 make hydrogen bonds with the backbone of the Asp951. The 2-NH$_2$ of 295 and 5-NH$_2$ of 298 make hydrogen bonds with the backbone of Leu899 and Glu948. The acidic groups extend to a solvent exposed pocket, where the α-COOH interacts via an ionic
interaction with Arg871 and the γ-COOH with Arg897 and Lys844 via water molecules. The docked scores for 295 and 298 in GARFTase were -14.28 and -14.33 kcal/mol, respectively.

**Figure 89.** A. Molecular modeling of 295 (magenta) with human AICARFTase (PDB ID: 1P4R). B. Molecular modeling of 298 (green) with human AICARFTase (PDB ID: 1P4R).

Figure 89 shows the molecular modeling of 295 (magenta) and 298 (green) in AICARFTase. Similar hydrogen bonds were observed with the scaffolds of 295 and 298 in the active site. The 2-NH$_2$ of 295 and 5-NH$_2$ of 298 hydrogen bond interaction with the Asn489 and an ion-dipole interaction with the Asp546. The 4-oxo of 295 and 7-oxo of 298 hydrogen bonds with Asn547. The scaffold is stabilized by a π–π stacking interactions with Phe544. The acidic functional groups for 295 and 298 show different orientations. For 295, the α-COOH forms an ion-dipole interaction with Ser565 and the backbone of Ala566. The γ-COOH of 131 is exposed to the solvent area. On the other hand, the α-COOH of 298 forms hydrogen bond with water, and γ-COOH is exposed to the solvent. The docked scores for 295 and 298 in AICARFTase were -10.76 and -10.68 kcal/mol, respectively.
Figure 90: Superposition of docked poses of 5-CHO-THF (yellow) and 295 (magenta) in the SHMT2 folate bonding site (PDB ID: 5V7I)\textsuperscript{389}

The natural cofactor 5-CHO-THF can be used to determine the binding requirements at the SHMT2 binding site. To determine the structural requirements for binding to the SHMT2 at folate binding site, 5-CHO-THF and 295 (pyrrolo[3,2-\textit{d}]pyrimidine) were docked in the recently published SHMT2 crystal structure.\textsuperscript{389} Figure 90 shows the docked pose of 5-CHO-THF (yellow) and 295 in the SHMT2 binding site. The folate active site is located at the interface of chain A (pink) and chain B (purple) and consists of a cavity at the bottom with a narrow channel opening into the solvent-exposed region. The pteridine ring of 5-CHO-THF binds to the cavity and the para-amino benzoyl (PABA) ring binds to the narrow channel lined by Tyr176 of chain B and Tyr105 of chain A. The $\alpha$-COOH makes a salt bridge with Lys181 (chain B) whereas the $\gamma$-COOH makes an ion-dipole interaction with Ser178 (chain B). The docked pose reveals a conformation with the pteridine ring orthogonal to the PABA ring. Based on the docked pose of 5-CHO-THF, it
was hypothesized that, relative to the PABA ring, an orthogonal conformation of the scaffold is favorable to bind to the SHMT2 binding site. The docked score of 5-CHO-THF in SHMT2 was -10.42 kcal/mol.

Figure 90 shows the superposition of 295 and 5-CHO-THF in the SHMT2 binding site where the pyrrolo[3,2-\textit{d}]pyrimidine scaffold binds deeper into the pocket similar to 5-CHO-THF. This allows the phenyl side chain of 295 to make $\pi$–$\pi$ interactions with Tyr176 (chain B) and Tyr105 (chain A). The $\alpha$-COOH group make salt bridges with Lys181 (chain B) and $\gamma$-COOH penetrates the solvent area. The docked pose of the 5-substituted pyrrolo[2,3-\textit{d}]pyrimidine analog 291 does not bind deep into the SHMT2 binding pocket as the 5-CHO-THF (Figure not shown). Compound 295 (-9.93 kcal/mol) showed a better docked score than 291 (-8.21 kcal/mol). Moreover, 295 displayed the orthogonal conformation similar to 5-CHO-THF. This suggests that compounds with the pyrrolo[3,2-\textit{d}]pyrimidine scaffold would be a better inhibitor of SHMT2 than those with the pyrrolo[2,3-\textit{d}]pyrimidine.

Compound 298 (Figure 85: Series XVIII) with a pyrazolo[4,3-\textit{d}]pyrimidine was designed to probe the effect of an isosteric replacement of C-2 in a pyrrolo[3,2-\textit{d}]pyrimidines 294-296 to N-2. The proposed compound 298 will evaluate the importance of HBA at the N-2-position and the additional nitrogen atom at the 2-position will help to determine the binding of the pyrazolo[4,3-\textit{d}]pyrimidine ring system in the SHMT2 binding site.
**Figure 91:** A: Molecular modeling studies of 295 (magenta) in the SHMT2 folate bonding site (PDB ID: 5V7I); B: Molecular modeling studies of 298 (green) in the SHMT2 bonding site (PDB ID: 5V7I).  

To better understand the binding of 295 and 298 with the folate SHMT2 binding site, both the compounds were docked in the SHMT2 binding site (PDB ID: 5V7I) (Figure 91). The 2-NH$_2$ and 3-NH of 295 make hydrogen bonds with the backbone of Pro167 (chain B). The 5-NH$_2$ and 6-NH of 298 make hydrogen bonds with the backbone of Gly170 (chain B). The α- and γ-COOH groups make salt bridges with Lys181 (chain B) and Lys103 (chain A), respectively. The docked score of 295 and 298 in the SHMT2 folate binding site were -9.93 and -10.55 kcal/mol, respectively.
Docked poses of the 5-CHO-THF (yellow), 295 (magenta), and 298 (green) were superposed (Figure 92). Both compounds 295 and 298 with a side chain phenyl ring can mimic the orthogonal conformation of 5-CHO-THF. This unique feature of the pyrrolo[3,2-d]pyrimidine and pyrazolo[4,3-d]pyrimidine scaffold could be the reason that 295 and 298 can bind into the cavity of the SHMT2 binding site. Therefore the pyrazolo[4,3-d]pyrimidine scaffold offers a unique opportunity to develop dual one-carbon metabolism inhibitors with inhibition of cytosolic de novo purine biosynthesis enzymes and the mitochondrial SHMT2 enzyme. Such compounds with selective transport via FRs and/or PCFT over RFC can achieve targeted tumor therapy with multiple enzyme inhibitions that would eliminate or decrease dose-limiting toxicity and would circumvent or delay the development of tumor resistance.
Figure 93. Superposition of docked pose of 298 in the crystal structures of FRα\textsuperscript{190} (cyan), FRβ\textsuperscript{388} (orange), GARFTase\textsuperscript{211} (green), AICARFTase\textsuperscript{219} (grey) and SHMT2 (magenta).\textsuperscript{389}

The docking studies of 298 in the targets FRα, FRβ, GARFTase, AICARFTase, and SHMT2 (Figures 86-89 and 92) displayed different conformational preferences of the side chain linker with reference to the pyrazolo[4,3-\textit{d}]pyrimidine scaffold. Superimposition of the scaffold of the docked poses of 298 were carried out in the crystal structures for FRs, GARFTase, AICARFTase and SHMT2 that enabled visualization of these relative side chain conformations (Figure 93). In reference to the scaffold, the side chains extend at different angles to bind to a particular active site. It emphasizes the importance of the necessity of a flexible linker, with a specific length to obtain optimal activity at all four targets to preserve the multitargeted attributes of these agents.
C.4.2. Design of \(N2\)-substituted 5-amino-2,6-dihydro-7\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-ones

**Table 21.** IC\(_{50}\) Values (nM) for 6-Substituted Pyrrolo[2,3-\(d\)]pyrimidine Classical Antifolates in RFC-, PCFT-, and FR-Expressing Cell Lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R2</th>
<th>RFC</th>
<th>FR(\alpha)</th>
<th>FR(\beta)</th>
<th>PCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC43-10</td>
<td></td>
<td></td>
<td>R2/hPCFT4</td>
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<tr>
<td>299</td>
<td>&gt;1000</td>
<td>649(38)</td>
<td>6.3(1.6)</td>
<td>5.6(1.2)</td>
<td>23.0(3.3)</td>
</tr>
<tr>
<td>300</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>6.3(1.6)</td>
<td>10(2)</td>
<td>213(28)</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60(8)</td>
<td>13.2(2.4)</td>
</tr>
</tbody>
</table>

**Table 22:** Growth inhibition assays: IC\(_{50}\) Values (nM) for 6-Substituted Pyrrolo[2,3-\(d\)]pyrimidines in KB human tumor sublines (expressing RFC, FR\(\alpha\), and PCFT) and protection study results by added metabolites.

<table>
<thead>
<tr>
<th></th>
<th>KB</th>
<th>KB (+Thd/Ade/AICA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>299</td>
<td>1.7(0.4)</td>
<td>Ade/AICA</td>
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<tr>
<td>300</td>
<td>1.9(0.7)</td>
<td>Ade/AICA</td>
</tr>
<tr>
<td>PMX</td>
<td>68 (12)</td>
<td>Thd/Ade</td>
</tr>
</tbody>
</table>

Gangjee et al.\(^{390}\) reported novel 6-substituted classical pyrrolo[2,3-\(d\)]pyrimidines 299-300 as cytotoxic antifolates with varying lengths of the carbon bridge (Table 21-22) and are
characterized by having selective FRα and FRβ transport over RFC. The three- and four-carbon bridged analogs 299 and 300 were active toward FR-expressing human tumors (KB and IGROV1) and the cytotoxicity was primarily due to potent inhibition of GARFTase, the first folate-dependent reaction in de novo purine nucleotide biosynthesis. The three-carbon pyrrolo[2,3-d]pyrimidine derivative, 299 (Table 21), was subsequently reported to also be a substrate for PCFT, thus providing an additional means of tumor-targeting.391

Figure 94: Series XIX

In an attempt to optimize the structure for selective transport by FR and/or PCFT, and GARFTase inhibitory activity, the pyrazolo[4,3-d]pyrimidines 301-302 with N-2 substitutions with the three- and four-carbon bridge analogs (Series XIX, Figure 94) were designed by selecting 6-substituted pyrrolo[2,3-d]pyrimidines 299-300 as the lead compounds and scaffold hopping approach.

To understand the binding of these compounds in transporters and intracellular targets, molecular modeling studies of 299-302 were carried out in crystal structures of FRs,190,388 GARFTase,211 AICARFTase219 and SHMT2389 using Schrödinger Maestro.156 The docked scores of the proposed compounds in all the five targets (FRα, FRβ, GARFTase, AICARFTase, SHMT2) were similar to that obtained for their respective lead compounds (±1.0 kcal/mol).
**Figure 95.** A: Molecular modeling of docked pose of 300 (violet) with human FRα; 95. B: Molecular modeling of docked poses 302 (orange) with human FRα (PDB ID: 5IZQ)\(^{190}\)

**Figure 96.** A: Molecular modeling of docked pose of 300 (cyan) with human FRβ; 96. B: Molecular modeling of docked pose of 302 (orange) with human FRβ (PDB ID: 4KN2)\(^{388}\)

Figure 95 depicts the binding mode of 300 and 302 in the active site of FRα. The 2-NH\(_2\) of 300 and 5-NH\(_2\) of 302 hydrogen bond interactions with Asp81. The 4-oxo of 300 and 7-oxo of 302 makes ion-dipole interactions with Arg103 and Arg106. The 3-NH of 300 and 6-NH of 302 make a water mediated hydrogen bond with Arg106. Amino acids Tyr85 and Trp171 stabilize both the
scaffold. In compound 302 the α-COOH forms a salt bridge with Lys136, and the γ-COOH forms an ion-dipole interaction with Trp140. The docked scores for 300 and 302 in FRα were -15.16 and -15.32 kcal/mol, respectively. This suggests that 302 would display a transport similar to 300 via FRα.

Figure 96 demonstrates the molecular modeling studies of 300 and 302 in FRβ. The scaffold is stabilized by π–π stacking interaction with Trp187 and Tyr101. The 2-NH₂ of 300, 5-NH₂ of 302 and 4-oxo of 300, 7-oxo of 302 of both the compounds’ hydrogen bond with Asp97 and Arg119, respectively. The 3-NH of 300 and 6-NH of 302 make a water mediated hydrogen bond with Arg122. The α-COOH forms an ion-dipole interaction with Trp156 and the γ-COOH forms ionic interactions with Arg152 and an ion-dipole interaction with Gln116. The docked scores for 300 and 302 in FRβ were -14.15 and -14.86 kcal/mol, respectively. This suggest that 302 would display transport similar to 300 via FRβ.

![Figure 97](image_url)

*Figure 97: A: Molecular modeling of 300 (cyan) with human GARFTase; **B: Molecular modeling of 302 (orange) with human GARFTase (PDB ID: 4ZZI).* 211 5'-phosphoribosyl-glycinamide (GAR) depicted in yellow.
Figure 97 shows the molecular modeling studies of 300 and 302 in GARFTase. Scaffolds for both compounds display similar hydrogen-bonding interactions in the active site. The 2-NH$_2$ of 300 and 5-NH$_2$ of 302 hydrogen bonds with the backbone of Leu899 and Glu948. The 4-oxo of 300 and 7-oxo of 302 group hydrogen bonds with Asp951. The acidic functional groups are extended in a solvent-exposed pocket, where the α-COOH interacts via an ionic bonding with the Arg871 and the γ-COOH ionic bonds to Arg897 and Lys844 via water. The docked scores for 300 and 302 in GARFTase were -14.60 and -14.68 kcal/mol, respectively.

![Figure 97](image1)

**Figure 98.** A: Molecular modeling of 300 (cyan) with human AICARFTase; 98. B. Molecular modeling of 302 (orange) with human AICARFTase (PDB ID: 1P4R)$^{219}$

Figure 98 shows the molecular modeling of 300 and 302 in AICARFTase. The 2-NH$_2$ of 300 and 5-NH$_2$ of 302 form an ion-dipole interaction with Asp546. The 4-oxo of 300 and 7-oxo of 302 form a hydrogen bond with Asn547. The scaffold is stabilized by a π–π stacking interaction with Phe544. For 302, the α-COOH interacts via an ion-dipole interaction with Ser565 and the backbone of Ala566 and the γ-COOH is exposed to the solvent area. The docked scores for 300 and 302 in AICARFTase were -10.43 and -10.59 kcal/mol, respectively.
Figure 99: Superposition of docked poses of 298 (green) and 302 (magenta) in the SHMT2 folate binding site (PDB ID: 5V7I).\textsuperscript{389}

Figure 100: Superposition of docked poses of 5-CHO-THF (cyan), 298 (green) and 302 (magenta) in the SHMT2 folate bonding site (PDB ID: 5V7I).\textsuperscript{389}

Side chain phenyl ring attach to the N-1 of 298 (N-1 substituted pyrazolo[4,3-\textit{d}]pyrimidine) can mimic the orthogonal conformation of 5-CHO-THF (Figure 99, 100) for attachment to SHMT2. The side chain phenyl ring at the N-2 of 302 (N-2 substituted pyrazolo[4,3-\textit{d}]pyrimidine)
is unable to adopt the same conformation (Figure 99, 100) at SHMT2. Superimposition of the
docked poses of 5-CHO-THF (cyan), 298 (green), and 302 (magenta) clearly show the difference
(Figure 100). This unique feature of the N-1 substituted pyrazolo[4,3-\(d\)]pyrimidine scaffold could
be the reason that the proposed compound 298 can bind to the cavity of the SHMT2 binding site
and 302 cannot position itself in the cavity of SHMT2 as the natural 5-CHO-THF. The docked
scores of 298, 302, and 5-CHO-THF in SHMT2, are -10.55, -9.48, and -10.42 kcal/mol, respectively.

C.4.3. Design of 6-substituted 2-amino-3,7-dihydro-4\(H\)-pyrrolo[2,3-\(d\)]pyrimidin-4-ones

Table 23. IC\(_{50}\) Values (nM) in RFC-, PCFT-, and FR-Expressing Cell Line and in KB human
tumor sublines (expressing RFC, FR\(\alpha\), and PCFT).\(^{190}\)

<table>
<thead>
<tr>
<th></th>
<th>R2</th>
<th>RFC</th>
<th>FR(\alpha)</th>
<th>FR(\beta)</th>
<th>PCFT</th>
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<td></td>
</tr>
<tr>
<td>300</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>6.3(1.6)</td>
<td>10(2)</td>
<td>213(28)</td>
<td>1.9(0.7) Ade/AICA</td>
</tr>
<tr>
<td>303</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>4.6 (1.3)</td>
<td>5.6(1.4)</td>
<td>&gt;1000</td>
<td>2.5(0.64) Ade/AICA</td>
</tr>
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<td>304</td>
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<td>&gt;1000</td>
<td>0.58(0.12)</td>
<td>1.6(0.44)</td>
<td>23(2)</td>
<td>0.59 Ade/AICA</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60 (8)</td>
<td>13.2(2.4)</td>
<td>68 (12) Thd/Ade</td>
</tr>
</tbody>
</table>
Table 24. Distances and bond angle variations measured for energy-minimized conformations of 300 (X =CH$_2$) and 303 (X=O) predicted by Schrodinger Maestro.$^{156}$

<table>
<thead>
<tr>
<th></th>
<th>X</th>
<th>C-X Bond distance (Å)</th>
<th>C-X-C Bond angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>CH$_2$</td>
<td>1.52</td>
<td>112.8</td>
</tr>
<tr>
<td>303</td>
<td>O</td>
<td>1.38</td>
<td>119.4</td>
</tr>
</tbody>
</table>

Gangjee and coworkers$^{390}$ reported compound 300 (Table 23) which showed selective PCFT and FRs cellular uptake over RFC and inhibited GARFTase, resulting in cytotoxicity and inhibition of tumor cell proliferation. Golani et al.$^{190}$ reported the 6-substituted pyrrolo[2,3-d]pyrimidine 303 (Table 23) with a four-atom chain linker as transporter-specific inhibitors of GARFTase. Compound 303 was designed with a heteroatom “O” vicinal to the phenyl ring. The design is based on the natural substrate for GARFTase (10-CHO-THF), which has a -CH$_2$N- two-atom bridge on a 6,6-pteridine system. The analog 303 with an oxygen hetero atom linker showed comparable potency with 300 toward KB human tumor cells. Compound 303 displayed 1.5- and 2-fold better activities in FR$\alpha$-expressing RT16 cells and FR$\beta$ expressing D4 cells, respectively than the lead analog 300. Anti-proliferative activities toward PCFT- (R2/PCFT4) expressing cells were undetectable for 303 compared to 300. The improvement in activity in FR$\alpha$-expressing RT16 cells and FR$\beta$ expressing D4 cells for 303 was attributed to chain length variation (due to varying C-X bond distances) and C-X-C bond angles. The bond lengths of C-X follow the trend: C-O < C-CH$_2$.$^{156}$ The C-X-C bond angle follows the trend: C-CH$_2$-C < C-O-C (Table 24).$^{156}$ Thus, replacing
the benzylic CH₂ in 300 with an oxygen in 303 decreases the bond length and increase the bond angle, which allows a different conformation of the side-chain phenyl ring to better accommodate in the active site of FRα and FRβ. These structural alterations could impact transport specificity and GARFTase inhibition, and it was of interest to determine how these variations would affect biological activity.392

Compound 304 (Table 23) with a fluorine at the 2′-position of 300 was designed and reported by Ravindra et al.393 Introduction of a fluorine can have direct (amphiphatic character, resulting in polar and hydrophobic interactions) or indirect (metabolism, lipophilicity, changes in acidity and basicity, etc.) effects on the lead molecules.393 The 2′-fluoro substitution in 304 dramatically increased antiproliferative activity mediated through all three transporters, with the most significant impact (11- and 9-fold, respectively) on the FRα and PCFT-expressing CHO cell lines compared to 300. Compounds 304 also inhibited the proliferation of IGROV1 and A2780 epithelial ovarian cancer cells; in IGROV1 cells with knockdown of FRα.393 Compound 304 also inhibited GRAFTase, a key enzyme in the de novo purine biosynthesis pathway.393

Figure 101. Series XX
Compound 305 (Series XX, Figure 101) was designed as analog of 303 and 304. Based on the biological evaluations of 300 and 303-304 (Table 19), compound 305 was designed to evaluate and compare the effects of replacing the benzylic 11-CH$_2$ of 304 with an oxygen to compare its effects towards improving selectivity in FRα, FRβ and PCFT over RFC.

![Figure 102](image)

**Figure 102.** A. Molecular modeling studies of docked poses of 304 (pink) and 305 (green) with human FRα (PDB ID: 5IZQ)\textsuperscript{190}; 102. B. Molecular modeling studies of docked poses of 304 (pink) and 305 (green) with human FRβ (PDB ID: 4KN2)\textsuperscript{388}

Figure 102A depicts the binding mode of 304 and 305 in the active site of FRα. For both compounds, the 2-NH$_2$ forms a hydrogen bond with Asp81. The 4-oxo makes ion-dipole interactions with Arg103 and Arg106. The 3-NH makes a water mediated hydrogen bond with Arg106. Amino acids Tyr175 and Trp171 stabilize the scaffold. The α-COOH forms an ion-dipole interaction with Trp140 and the γ-COOH forms a salt bridge with Lys136 and ion-dipole interaction with Trp102. The docked scores for 304 and 305 in FRα were -15.22 and -15.16 kcal/mol, respectively. This suggests that 305 would display transport similar to 304 via FRα.
Figure 102B demonstrates the molecular modeling of 304 and 305 in FRβ. The scaffold is stabilized by π–π stacking interactions with Trp187 and Tyr101. The 2-NH$_2$ group of both compounds’ hydrogen bond with Asp97. The 4-oxo makes ion-dipole interactions with Arg119 and Arg122. The 3NH makes a water mediated hydrogen bond with Arg122. The α-COOH forms ion-dipole interaction with Trp156 and the γ-COOH forms ionic interaction with the backbone of Arg119 and an ion dipole interaction with Trp118. The docked scores for 304 and 305 in FRβ were -15.11 and -15.10 kcal/mol, respectively. This would suggest that 305 would display similar transport through FRβ.

**Figure 103.** A. Molecular modeling studies of docked pose of 304 (pink) with human GARFTase; B. Molecular modeling studies of docked pose of 305 (green) with human GARFTase. 5′-phosphoribosyl-glycinamide (GAR) depicted in yellow.

Figures 103A and 103B show the molecular modeling of 304 and 305 in GARFTase, respectively. Scaffolds for both compounds display similar hydrogen-bond interactions in the active site. The 4-oxo of both the compounds interact with the backbone of the Asp951. The 2-NH$_2$ for both the compounds make hydrogen bonds with the backbone of Leu899 and Glu948. The
3-NH makes a hydrogen bond with the backbone of Ala947. The N1 of the scaffold makes a hydrogen bond with the backbone of Ile898. The 5′-phosphoribosyl-glycinamide (GAR) depicted in yellow makes hydrogen bond with the oxygen of 305 (Figure 103B). This type of interaction is absent of 304 with -CH_2- the C-11 position. The acidic functional groups are extended to a solvent-exposed pocket, where the α-COOH interacts via an ionic bonding with Arg871 and γ-COOH with the Arg897 and Lys844 via a water molecule. The docked scores for 304 and 305 in GARFTase were -15.12 and -15.38 kcal/mol, respectively.

**Figure 104.** A. Molecular modeling of docked pose of 304 (pink) with human AICARFTase; 104. B. Molecular modeling of docked pose 305 (green) with human AICARFTase (PDB ID: 1P4R)^219

Figures 104A and 104B show the molecular modeling of 304 and 305 in AICARFTase, respectively. Scaffolds of 304 and 305 display similar hydrogen bond with the active site. The 2-NH_2 forms a hydrogen bond interaction with Asn489 and an ion-dipole interaction with Asp546. The 4-oxo forms a hydrogen bond interaction with Asn547. The 3-NH makes an ion dipole interaction with Asp546. The scaffolds are stabilized by a π–π stacking interaction with Phe544 and Phe315. The acidic functional groups for 304 and 305 show different orientations. For 304,
the α-COOH makes ion-dipole interactions with a water molecule and the γ-COOH makes an ionic interaction with Lys357. On the other hand, the α-COOH of 305 forms an ion-dipole interaction with water molecule and the γ-COOH is exposed to the solvent. The docked scores for 304 and 305 in AICARFTase were -11.16 and -11.29 kcal/mol, respectively.

![Figure 105: Superposition of the scaffold docked pose of 305 in crystal structures of FRα (cyan), FRβ (orange), GARFTase (green) and AICARFTase (grey).](image)

The docking studies of 305 in the targets (Figures 102-104) displayed different conformational preferences of the side chain linker in reference to the pyrrolo[2,3-\(d\)]pyrimidine scaffold. Superimposition of the docked poses of 305 was carried out in the crystal structures for FRs, GARFTase, and AICARFTase enabled visualization of these conformations (Figure 105). In reference to the scaffold, the side chains extend at different angles to bind to a particular active site. It emphasizes the importance of the necessity of a flexible linker, with a specific length to obtain optimal activity at all four targets.
IV. CHEMICAL DISCUSSION

The present section deals with the development of synthesis for compounds in the following four areas:

D.1. Synthesis of agents with combination chemotherapy potential in single agents

4) 2, 4-disubstituted thieno[2,3-\(d\)]pyrimidines
5) 2, 4, 5- and 6-substituted thieno[2,3-\(d\)]pyrimidines
6) 4, 7-disubstituted pyrrolo[3,2-\(d\)]pyrimidines


3) 2, 4-disubstituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidine
4) 2, 4, 5-trisubstituted pyrazolo[3,2-\(d\)]pyrimidines

D.3. Synthesis of selective pJDHFR inhibitors

2) 6-(aryltio)pyrido[2,3-\(d\)]pyrimidine-2,4-diamines

D.4. Synthesis of single agents with Folate Receptors (FRs) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one carbon (1C) metabolism inhibitors

4) \(N1\)-substituted 5-amino-1,6-dihydro-7\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-ones
5) \(N2\)-substituted 5-amino-2,6-dihydro-7\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-ones
6) 6-substituted 2-amino-3,7-dihydro-4 -pyrrolo[2,3-\(d\)]pyrimidin-4-ones
D.1. Synthesis of agents with combination chemotherapy potential in single agents

D.1.1: Synthesis of 2-, 4-, 5- and 6-substituted thieno[2,3-\(d\)]pyrimidines

Scheme 46. Synthesis of intermediate \(\text{308}\)

Reagents and conditions: a) HCl (g), 30 mins, CH\textsubscript{3}CN, rt, 2 h; b) POCl\textsubscript{3}, 12-24 h; c) POCl\textsubscript{3}, pyridine, toluene, reflux, 12h.

The synthesis of the bicyclic thieno[2,3-\(d\)]pyrimidine \(\text{307}\) (Scheme 46) was accomplished by the treatment of commercially available ethyl 2-aminothiophene-3-carboxylate \(\text{306}\) with acetonitrile under acidic condition.\(^{293}\) To synthesize 4-chloro-2-methylthieno[2,3-\(d\)]pyrimidine \(\text{308}\), chlorinating agent POCl\textsubscript{3} was added to \(\text{307}\) at reflux for 12-24 h. The reaction was cooled, excess chlorinating agent was evaporated and neutralized with the ammonia solution, and the mixture was filtered. During work up, the 4-chloro product \(\text{308}\) converted back to the 4-oxo product \(\text{307}\) (confirmed by \(^{1}\text{H NMR}\)). Other chlorinating agents like thionyl chloride (SOCl\textsubscript{2})\(^{394}\) or phosphorus pentachloride (PCl\textsubscript{5})\(^{395}\), or a mixture of POCl\textsubscript{3} and PCl\textsubscript{5}\(^{396}\) were attempted to convert \(\text{307}\) to \(\text{308}\). In all of these cases, where these chemicals were utilized as both chlorinating agents and solvents, the excess of the solvent removal and the work-up process was very tedious.
**Table 25: Chlorination conditions to covert 307 to 308**

<table>
<thead>
<tr>
<th>No.</th>
<th>Chlorinating agents</th>
<th>Base</th>
<th>Status of reaction/yields%</th>
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<td>4.</td>
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</tbody>
</table>

Sun *et al.* superscript 397 reported a method where nitrogen containing aromatic heterocyclic analogs were efficiently prepared from the corresponding hydroxylated precursors under low-solvent conditions with equimolar or less chlorinating reagents. Arnott *et al.* superscript 398 also described methods for chlorination of 4-quinazolones using POCl₃. This high yielding protocol allows successful preparations of multigram and kilogram quantities of critical synthetic intermediates. The chlorination reaction was attempted at reflux with POCl₃ (1 eq.) and with several different combinations of solvents (dioxane, acetonitrile, toluene) and bases (1 eq.) (Table 25). Optimized results were obtained at reflux for 12 h with POCl₃ (1 eq.) and pyridine (1 eq.) in toluene to afford 308 in 62% yield.

**Scheme 47. Synthesis of target compounds 182-184.**

![Scheme 47](image)

Reagents and conditions: a) anilines, isopropanol, 1 drop of HCl, microwave, 120 °C, 6 h; b) sodium hydride, iodomethane, DMF, r.t., 2 h.
To afford a nucleophilic aromatic substitution reaction,\textsuperscript{309} \textbf{308} was heated in a microwave reactor at 120 °C with 4-methoxyaniline or 4-(methylthio)aniline to afford \textbf{183} and \textbf{184a}, respectively (Scheme 47). The \textit{N}\textsuperscript{4}-methylation was carried out using the published procedure\textsuperscript{346} with sodium hydride and iodomethane in DMF with \textbf{183} and \textbf{184a} to afford \textbf{182} and \textbf{184} in 78%, and 52% yield, respectively.

**Scheme 48. Synthesis of target compound \textbf{185}**

![Scheme 48](image)

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, 1 drop HCl, isopropanol, 120 °C, microwave, 6 h.

Following the reported method,\textsuperscript{293} 4-(6-methoxy-3,4-dihydroquinolin-1(2\textit{H})-yl)-2-methylthieno[2,3-\textit{d}]pyrimidine \textbf{185} (Scheme 48) was synthesized using 4-chloro-2-methylthieno[2,3-\textit{d}]pyrimidine \textbf{308} and 6-methoxy-1,2,3,4- tetrahydroquinoline with a drop of HCl in isopropanol at 120 °C in a microwave reactor for 6 h (76% yield).

**Scheme 49. Synthesis of the target compound \textbf{189}**

![Scheme 49](image)

Reagents and conditions: a) 6-aminonaphthalen-1-ol, isopropanol, 1 drop HCl, microwave, 120 °C, 8 h; b) sodium hydride, iodomethane, DMF, r.t., 2 h.
Intermediate 308 and 6-aminonaphthalen-1-ol were dissolved in isopropanol and heated in a microwave reactor at 120 °C using the reported method\textsuperscript{293} followed by methylation of the \textit{N}^4-H and 5'-OH with sodium hydride and iodomethane in DMF solution to afford 189 in 48% yield over two steps (Scheme 49).\textsuperscript{346}

**Scheme 50. Synthesis of target compound 196**

\[
\begin{array}{c}
309 \quad \xrightarrow{a} \quad 196 (60\%)
\end{array}
\]

**Reagents and conditions:**

a) 4-methoxy-\textit{N}-methylaniline, isopropanol, 1 drop of HCl, microwave, 120 °C, 6 h.

The synthesis of \textit{N}-(4-methoxyphenyl)-\textit{N}-methylthieno[2,3-\textit{d}]pyrimidin-4-amine 196 (Scheme 50) was carried out with the reported S\textit{N}Ar reaction\textsuperscript{399} in isopropanol with treatment of commercially available 4-chlorothieno[2,3-\textit{d}]pyrimidine 309, 1 drop of HCl and 4-methoxy-\textit{N}-methylaniline in a microwave reactor for 6 h (60% yield).

**Scheme 51. Synthesis of target compound 198**

\[
\begin{array}{c}
309 \quad \xrightarrow{a} \quad 198 (58\%)
\end{array}
\]

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, 1 drop of HCl, isopropanol, microwave, 120 °C, 8 h.
Commercially available 4-chlorothieno[2,3-\(d\)]pyrimidine 309 was treated with 1 drop of HCl followed by the addition of 6-methoxy-1,2,3,4-tetrahydroquinoline and heated in a microwave reactor to afford 4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)thieno[2,3-\(d\)]pyrimidine 198 in 58% yield (Scheme 51).

**Scheme 52. Synthesis of intermediate 311**

![Diagram](image)

Reagents and conditions: a) Chloroformamidine hydrochloride, methyl sulfone, 140 °C, 4 h; b) Piv\(_2\)O, reflux, 2 h; c) POCl\(_3\), pyridine, toluene, reflux, 10 h

Commercially available ethyl 2-aminothiophene-3-carboxylate 306 was cyclized to 2-aminothieno[2,3-\(d\)]pyrimin-4(3\(H\))-one 310 with chloroformamidine hydrochloride in dimethyl sulfone (Scheme 52). Intermediate 310 was protected with pivalic anhydride and chlorinated with POCl\(_3\) and pyridine in toluene to afford the crucial intermediate 311 (49% over two steps).

**Scheme 53. Synthesis of target compound 197**

![Diagram](image)

Reagents and conditions: a) 4-methoxy-N-methylaniline, isopropanol, 1 drop HCl, reflux, 12 h.; b) 1 N NaOH, reflux, 2 h.
For the synthesis of \( N4-(4\text{-methoxyphenyl})-N4\text{-methylthieno}[2,3-\text{d}]\text{pyrimidine-2,4-diamine} \) 197 (Scheme 53), the displacement method was followed in the first step as previously reported\(^{400}\) by treating 311 with 4-methoxy-\( N \)-methylaniline at reflux condition for 12 h. The pivaloyl protected intermediate was deprotected with 1 N NaOH and heated to reflux for 2 h to obtain 197 in 48% yield over two steps.

Scheme 54. Synthesis of target compound 199

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, isopropanol, 1 drop of HCl, reflux, 8 h.; b) 1 N NaOH, reflux, 2 h.

For the synthesis of 4-(6-methoxy-3,4-dihydroquinolin-1(2\( H \))-yl)thieno[2,3-\text{d}]pyrimidin-2-amine 199 (Scheme 54), the displacement method as previously reported by Gangjee and co-workers\(^{353}\) was followed where 311 was treated with 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline in isopropanol at reflux condition for 12 h. To crude reaction was added 1 N NaOH solution and maintained at reflux for 2 h to obtain 199 in 40% in two steps.
Scheme 55. Synthesis of the compound 204

![Scheme 55](image)

Reagents and conditions: a) 4-methoxy-N-methylaniline, isopropanol, r.t., 48 h.

Commercially available 2,4-dichlorothieno[2,3-d]pyrimidine 312 was reacted with 4-methoxy-N-methylaniline at r.t. for 48 h in acetonitrile in order to synthesize 2-chloro-N-(4-methoxyphenyl)-N-methylthieno[2,3-d]pyrimidin-4-amine 204 in 38% yield following the reported procedure by Shah and Gangjee\textsuperscript{353} (Scheme 55).

Scheme 56. Synthesis of compound 205

![Scheme 56](image)

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, isopropanol, r.t., 48 h.

Commercially available 2,4-dichlorothieno[2,3-d]pyrimidine 312 was reacted with 6-methoxy-1,2,3,4-tetrahydroquinoline at r.t. for 48 h in order to synthesize 2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)thieno[2,3-d]pyrimidine 205 in 35% yield following the reported procedure by Shah and Gangjee\textsuperscript{353} (Scheme 56).
D.1.2: Synthesis of 2, 4, 5- and 6-substituted thieno[2,3-d]pyrimidines

Scheme 57. Synthesis of target compound 218

Reagents and conditions: a) HCl (g) 30 mins, CH₃CN, rt, 1 h; b) POCl₃, pyridine, toluene, reflux, 12 h, c) 4-methoxy-N-methylaniline, isopropanol, 1 drop of HCl, reflux, 12 h

The synthesis of 2,5-dimethylthieno[2,3-d]pyrimidin-4(3H)-one 314 (Scheme 57) was accomplished by the treatment of commercially available ethyl 2-amino-4-methylthiophene-3-carboxylate 313 with acetonitrile under acidic conditions. Chlorination of 314 with POCl₃ and pyridine in toluene afforded the intermediate 4-chloro-2,5-dimethylthieno[2,3-d]pyrimidine 315a. Nucleophilic aromatic substitution reaction of the intermediate 315a with 4-methoxy-N-methylaniline in isopropanol with 1 drop of HCl afforded 218 in 62% yield.

Scheme 58. Synthesis of target compound 221

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, isopropanol, 1 drop of HCl reflux, 6 h.
Intermediate 315a was subjected to a S_NAr displacement with 6-methoxy-1,2,3,4-tetrahydroquinoline using the published procedure\textsuperscript{401} to synthesize 4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2,5-dimethylthieno[2,3-d]pyrimidine 221 in 60% yield (Scheme 58).

**Scheme 59. Synthesis of target compounds 219-220**

![Scheme 59](image)

315b, R = Cl  
315c, R = NH\textsubscript{2}  
219, R = Cl (48%)  
220, R = NH\textsubscript{2} (45%)

Reagents and conditions: a) 4-methoxy-N-methylaniline, isopropanol, 1 drop of HCl, r.t., 18-24 h.

Commercially available 2,4-dichloro-5-methylthieno[2,3-d]pyrimidine 315b and 4-chloro-5-methylthieno[2,3-d]pyrimidin-2-amine 315c were displaced with 4-methoxy-N-methylaniline following the reported procedure by Shah and Gangjee\textsuperscript{353} at r.t. for 18-24 h to generate 2-chloro-N-(4-methoxyphenyl)-N,5-dimethylthieno[2,3-d]pyrimidin-4-amine 219 and N4-(4-methoxyphenyl)-N4,5-dimethylthieno[2,3-d]pyrimidine-2,4-diamine 220 in 48% and 45% yield, respectively (Scheme 59).

**Scheme 60. Synthesis of target compounds 222-223**

![Scheme 60](image)

315b, R = Cl  
315c, R = NH\textsubscript{2}  
222, R = Cl (45%)  
223, R = NH\textsubscript{2} (40%)

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, isopropanol, 1 drop of HCl, r.t., 18-24 h
Commercially available 2,4-dichloro-5-methylthieno[2,3-\textit{d}]pyrimidine 315b and 4-chloro-5-methylthieno[2,3-\textit{d}]pyrimidin-2-amine 315c were displaced with 6-methoxy-1,2,3,4-tetrahydroquinoline following the reported procedure by Shah and Gangjee\textsuperscript{353} and at r.t. for 18-24 h to generate 2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-5-methylthieno[2,3-\textit{d}]pyrimidine 222 and 4-(6-methoxy-3,4-dihydroquinolin-1(2\textit{H})-yl)-5-methylthieno[2,3-\textit{d}]pyrimidin-2-amine 223 in 45% and 40% yield, respectively (Scheme 60).

**Scheme 61. Synthesis of target compounds 225, 226a, 226 and 227**

![Scheme 61](image)

Reagents and conditions: a) HCl (g) 30 min, CH\textsubscript{3}CN, rt, 1 h; b) POCl\textsubscript{3}, pyridine, toluene, reflux, 12 h, c) anilines, isopropanol, 1 drop of HCl, reflux, 12 h, d) iodomethane, NaH, DMF, rt, 2 h.

The synthesis of 2,5,6-trimethylthieno[2,3-\textit{d}]pyrimidin-4(3\textit{H})-one 317 (Scheme 61) was accomplished by treatment of commercially available ethyl 2-amino-4,5-dimethylthiophene-3-carboxylate 316 with acetonitrile under acidic conditions.\textsuperscript{293} Chlorination of 317 with POCl\textsubscript{3} and
Pyridine in toluene afforded 4-chloro-2,5,6-trimethylthieno[2,3-\textit{d}]pyrimidine 318. Nucleophilic aromatic substitution reactions\textsuperscript{309} of the intermediate 318 with 4-methoxyaniline and 4-(methylthio)aniline in isopropanol with 1 drop of HCl afforded 227 and 228, respectively. Synthesis of \(N\)-(4-methoxyphenyl)-\(N\),2,5-trimethylthieno[2,3-\textit{d}]pyrimidin-4-amine (225) and \(N\),2,5,6-tetramethyl-\(N\)-(4-(methylthio)phenyl)thieno[2,3-\textit{d}]pyrimidin-4-amine (226) were carried out by treatment of 227 and 228 with sodium hydride in DMF at room temperature followed by dropwise addition of iodomethane for 2 h in 69\% and 36\% yield, respectively.

\textbf{Scheme 62. Synthesis of target compounds 229}

\begin{center}
\includegraphics[width=\textwidth]{scheme62.png}
\end{center}

Reagents and conditions: a) 7-methoxy-3,4-dihydro-2\textit{H}-benzo[\textit{b}][1,4]oxazine, isopropanol, 1 drop of HCl, reflux, 8 h.

Intermediate 318 was subjected to \(S\text{\textsubscript{N}}\text{Ar}\) displacement with 7-methoxy-3,4-dihydro-2\textit{H}-benzo[\textit{b}][1,4]oxazine using a published procedure\textsuperscript{401} to synthesize 7-methoxy-4-(2,5,6-trimethylthieno[2,3-\textit{d}]pyrimidin-4-yl)-3,4-dihydro-2\textit{H}-benzo[\textit{b}][1,4]oxazine 229 in 58\% yield (Scheme 62).
D.1.3. Synthesis of 4,7-disubstituted pyrrolo[3,2-d]pyrimidine

Scheme 63. Synthesis of the target compound 232

```
Reagents and conditions: a) 3,3-dimethoxypropanenitrile, CH₃ONa, CH₃OH, r.t., overnight; b) 6N HCl, r.t., 30 mins; c) H₂, Pd/C 50 psi, r.t., 3 h; d) diethylamino malonate, CH₃COONa, CH₃OH, r.t., 16 h; e) CH₃ONa, CH₃OH r.t., 12 h, reflux, 3 h; f) formamide, µW, 170 °C, 12 h; g) POCl₃, pyridine, toluene, reflux, 4 h; h) 4-methoxy-N-methylaniline, isopropanol, 1 drop of HCl, reflux, 3 h.

Key intermediate methyl 3-amino-4-benzyl-1H-pyrrole-2-carboxylate 323 (Scheme 63) was synthesized following the reported procedure by Elliott et al.⁴⁴⁰ A mixture of benzaldehyde 319 and 3,3-dimethoxypropionitrile was added to a solution of NaOMe in MeOH to afford 2-(dimethoxymethyl)-3-phenylacrylonitrile 320. This was followed by the addition of 6N HCl to deprotect the acetal and provide 2-formyl-3-phenylacrylonitrile 321.⁴⁴⁰ Catalytic reduction⁴⁴⁰ of 321 to 2-benzyl-3-oxopropanenitrile 322, followed by cyclization of 322 with diethylamino malonate and CH₃COONa in methanol provided the key intermediate methyl 3-amino-4-benzyl-
1H-pyrrole-2-carboxylate 323. Cyclization$^{320}$ of 323 with formamide produced 7-benzyl-5H-pyrrolo[3,2-d]pyrimidin-4-ol 324. This was then subjected to chlorination reaction$^{320}$ with POCl$_3$ to obtain 7-benzyl-4-chloro-5H-pyrrolo[3,2-d]pyrimidine 325. Nucleophilic aromatic substitution with 325 and 4-methoxy-N-methylaniline in isopropanol gave the final compound 232 in 72% yield.


**Scheme 64:** Retrosynthetic analysis to 2,4-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine.

From a retrosynthetic point of view (Scheme 64), the general strategy was envisioned for the synthesis of 2,4-disubstituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidines. The tricyclic 328 could be derived from 329, which in turn could be synthesized from 330. Synthesis of 330 could be attempted from the commercially available 4-cyclohexanone 331, sulfur 332, and
ethyl cyanoacetate 333 through the Gewald reaction\textsuperscript{402} and cyclization. Thus, 4-chloro-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine 328 is the key intermediate for the synthesis of 2- and 4-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidines. Attempt of N\textsuperscript{4}-methylation from 326 with iodomethane and nucleophilic aromatic substitution (S\textsubscript{N}Ar) from 327 could be accomplished from variety of anilines.

\textbf{Scheme 65.} Synthesis of target compounds 234 and 236.

Reagents and conditions: a) S\textsubscript{8}, ethyl cyanoacetate, morpholine, EtOH, 60 °C, 12 h; b) CH\textsubscript{3}CN, HCl (g), 30 min, r.t.; overnight; c) POCl\textsubscript{3}, pyridine, toluene, reflux, 6 h.; d) anilines, isopropanol, 1-2 drops of HCl, reflux, 4-8 h.; e) sodium hydride, iodomethane, DMF, r.t., 2 h.

Commercially available cyclohexanone 331 and sulfur in ethanol were treated with ethyl cyanoacetate, followed by the dropwise addition of morpholine to obtain 330 (Scheme 65).\textsuperscript{403} The synthesis of the tricyclic thieno[2,3-d]pyrimidine 329 was accomplished by bubbling hydrochloric acid\textsuperscript{293, 404} in 330 in acetonitrile. The 2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-}
4-chloro-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine 328. Nucleophilic aromatic substitution\(^{293}\) of 328 using 4-methoxyaniline and 4-(methylthio)aniline in isopropanol with 1 drop of HCl afforded 334 and 335 in 40 and 41% yield, respectively. The \(N^{4}\)-methylation\(^{405}\) was carried out using sodium hydride and iodomethane with 334 and 335 to afford 234 and 236 in 68% and 70% yield, respectively.

**Scheme 66. Synthesis of target compound 238**

Reagents and conditions: a) 6-methoxy-1,2,3,4-tetrahydroquinoline, 1 drop of HCl, isopropanol, reflux, 6 h.

To a solution of isopropanol and 328, 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline were added and the mixture kept at reflux to provide the synthesis of 238 (Scheme 66) in 55% yield.\(^{293}\)
Scheme 67. Synthesis of target compounds 239-240

Reagents and conditions: a) 6-aminonaphthalen-1-ol, 1 drop of HCl, isopropanol, reflux, 8h; b) sodium hydride, iodomethane, DMF, r.t. 2 h

To a solution of isopropanol and 328 was added with 1 drop of HCl and 6-aminonaphthalen-1-ol and kept at reflux to afford 6-((2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)amino)naphthalen-1-ol 240 in 71% yield (Scheme 67). Methylation\(^4,\) of \(N^4\)-H and 5′-OH of 240 was carried out using sodium hydride and iodomethane in DMF to afford \(N\)-(5-methoxynaphthalen-2-yl)-\(N,2\)-dimethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine 239 in 57% yield.

Scheme 68. Synthesis of target compounds 244 and 245.

Reagents and conditions: a) chloroformamidine hydrochloride, DMSO\(_2\), 140 °C, 4 h; b) Piv\(_2\)O, reflux, 2 h; c) POCl\(_3\), pyridine, toluene, reflux, 4 h; d) i) 1-2 drops of HCl, isopropanol, anilines, reflux, 6-8 h; ii) 1N NaOH, reflux 2 h.
Using the reported procedure,\textsuperscript{320} the synthesis of 2-amino-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-4(3\(H\))-one \textbf{336} was initiated by cyclization of \textbf{330} with chlorformamidine hydrochloride in dimethyl sulfone at 140 \(^\circ\)C (Scheme 68). The amino group of \textbf{336} was protected using pivalic anhydride to afford N-(4-oxo-3,4,5,6,7,8-hexahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-2-yl)pivalamide \textbf{337}. Chlorination of \textbf{337} was carried out with phosphorus oxychloride, pyridine in toluene to generate N-(4-chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-2-yl)pivalamide \textbf{338}. Following a reported procedure,\textsuperscript{320} S\textsubscript{N}Ar reaction using N-methyl-4-methoxyaniline and N-methyl-4-(methylthio)aniline in isopropanol under reflux condition followed by deprotection of the pivalate group with 1N NaOH under reflux afforded N4-(4-methoxyphenyl)-N4-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidine-2,4-diamine \textbf{244} and N4-methyl-N4-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidine-2,4-diamine \textbf{245} in 56\% and 48\% yield, respectively.

\textbf{Scheme 69}. Synthesis of target compound \textbf{246}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{scheme69}
\caption{Synthesis of target compound \textbf{246}}
\end{figure}

Reagents and conditions: a) 1 drop of HCl, isopropanol, 6-methoxy-1,2,3,4-tetrahydroquinoline, reflux, 8 h; ii) NaOH, reflux 1h.
To a solution of the intermediate 338 in isopropanol, 1 drop of HCl, and 6-methoxy-1,2,3,4-tetrahydroquinoline were added and the mixture was kept at reflux. This was followed by pivalate deprotection with 1N NaOH at reflux to afford 4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-2-amine 246 in 42% yield (Scheme 69).

Scheme 70. Synthesis of target compounds 247 and 248.

Following a reported procedure intermediate 330 was cyclocondensed with formamide in a microwave reactor at 180 °C for 12 h to afford tricyclic 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one 339 (Scheme 70). Chlorination of 339 with phosphorus oxychloride and pyridine in toluene generated 4-chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine 340 which was then subjected to S_N_Ar reaction using the appropriate anilines in isopropanol at reflux to afford 247 and 248 in 68% and 70% yield, respectively.
D.2.2: Synthesis of 2,4,5-trisubstituted pyrazolo[3,2-d]pyrimidines

Scheme 71. Attempted synthesis of the intermediate 342a

Reagents and conditions: a) Methylation agents, K₂CO₃, solvent, r.t., 12-24 h.

Table 26: Reaction optimization with various methylation agents at different solvents with K₂CO₃ base at r.t. for 12-24 h.

<table>
<thead>
<tr>
<th>Attempts</th>
<th>Methylation agents</th>
<th>Solvent</th>
<th>342a (%Yield)</th>
<th>342b (%Yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dimethyl sulphate</td>
<td>MeOH</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
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<td>DMF</td>
<td>28</td>
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</tr>
<tr>
<td>3</td>
<td>dimethyl sulphate</td>
<td>Acetone</td>
<td>342b inseparable</td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>MeOH</td>
<td>26</td>
<td>52</td>
</tr>
<tr>
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<td>methyl tosylate</td>
<td>DMF</td>
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<td>54</td>
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<td>7</td>
<td>MeI</td>
<td>DMF</td>
<td>35</td>
<td>55</td>
</tr>
</tbody>
</table>
The synthesis of the intermediate 342a (Scheme 71) was initiated from commercially available methyl 4-nitro-1H-pyrazole-5-carboxylate 341. Initially both the regioisomers 342a and 342b formation was observed. In addition, the decreased isomer 342a was obtained in as one half as much as the isomer 342b. The reaction was thus attempted in several different solvents with various methylation agents, with temperature variations from r.t. to higher temperatures and at different time intervals (Table 26).406,407 The most efficient yield of 342a was with methyl iodide in the presence of K₂CO₃ in DMF, at r.t. This provided desired regioisomer 342a in 35% yield and the other isomer in 55% yield. The isomers 342a and 342b were separated via column chromatography and were structurally characterized by ¹H NMR and NOESY spectroscopy.

D.2.2.1: ¹H NMR Evidence for the structure of compounds 342a and 342b:

![Figure 106: NOESY NMR of intermediate 342a](image)
The regiochemical identification of the methylated intermediates 342a and 342b was carried out by $^1$H NMR\textsuperscript{407} and NOESY spectroscopy (Figure 106 and 107). In the $^1$H NMR (only $\delta$ values are included, spectra not shown), the chemical shifts of N1-CH$_3$ protons for 342a and 342b occurred as singlets at 4.02 and 3.95 ppm, respectively. It is noteworthy that the presence of the adjacent C5-carboxylate to the N1-CH$_3$ in compound 342a caused the N1-CH$_3$ protons of 342a to shift ~0.07 ppm downfield than the N1-CH$_3$ of 342b. The initial studies involved NMR and NOESY
(Nuclear Overhauser Enhancement Exchange Spectroscopy) which is one of the most direct ways to determine spatial proton-proton/proton-heteronucleus/heteronucleus-heteronucleus correlations within a molecule in the range of up to 5 Å or less.\textsuperscript{408} Intermediate 342a, can be identified as the methyl 1-methyl-4-nitro -1\textit{H}-pyrazole-5-carboxylate regioisomer as no strong NOE correlation was observed between the H3 proton and the N1-CH\textsubscript{3} protons (Figure 106). However, the regioisomeric 342b regioisomer, the methyl 1-methyl-4-nitro -1\textit{H}-pyrazole-3-carboxylate showed characteristic NOE correlation between H5 proton and N1-CH\textsubscript{3} protons (Figure 107). The observed H5 proton and N1-CH\textsubscript{3} cross peak (δ 8.95, 3.95) was dominated by scalar coupling effects, with evidence of only a NOE coupling proton contributing to it in 342b. This establishes the structure of 342b. For 342a the H3 proton occurs at δ 8.36 and for 342b the H5 proton occurs at δ 8.95. The probable deshielding effects of the H5 proton in 342b is due to the direct electron attracting effect of the C-5. Literature precedence on \textsuperscript{1}H NMR\textsuperscript{407}, crystal structure\textsuperscript{407} and the 2D-NMR from our studies confirms the structure of the regioisomers 342a and 342b as depicted in Scheme 71.

**Scheme 72.** Synthesis of target compound 251.

![Scheme 72](image)

Reagents and conditions: a) 10\% Pd/C, H\textsubscript{2}, MeOH, 40 psi, 1 h; b) urea, 180 °C, 2 h; c) POCl\textsubscript{3}, pyridine, toluene, 4 h, reflux, d) 4-methoxy-N-methylaniline, acetonitrile, reflux, 12 h.
Reduction of the nitro group\textsuperscript{385} of 342a, yielded 343 (Scheme 72), which was cyclocondensed with urea\textsuperscript{407} followed by chlorination with POCl\textsubscript{3} and pyridine in toluene to provide the key intermediate 5,7-dichloro-1-methyl-1\textit{H}-pyrazolo[4,3-\textit{d}]pyrimidine 344. Nucleophilic aromatic substitution\textsuperscript{335} of 344 was carried out using 4-methoxy-\textit{N}-methylaniline to provide 5-chloro-\textit{N}-(4-methoxyphenyl)-\textit{N},1-dimethyl-1\textit{H}-pyrazolo[4,3-\textit{d}]pyrimidin-7-amine 251 in 38\% yield.

\textbf{Scheme 73. Synthesis of target compound 252}

![Scheme 73](image)

Reagents and conditions: a) 4-methoxyaniline, acetonitrile, reflux, 8 h.

Nucleophilic aromatic substitution\textsuperscript{335} of 344 with 4-methoxyaniline to provide 5-chloro-\textit{N}-(4-methoxyphenyl)-1-methyl-1\textit{H}-pyrazolo[4,3-\textit{d}]pyrimidin-7-amine (252) in 45\% yield (Scheme 73).

**D.2.2.2: \textsuperscript{1}H NMR Evidence for Conformational Restriction in Compounds 251 and 252:**

\textsuperscript{1}H-NMR studies were carried out to confirm that the \textit{S}_\text{N}Ar reaction occurred regiospecifically at the 7-position of 344 in Scheme 72 and Scheme 73 to generate 251 and 252, respectively. Comparing the chemical shift positions of the protons at the N1-CH\textsubscript{3} for 251 with the chemical shift of the protons at the N1-CH\textsubscript{3} for 252, it was observed that the protons after the displacement are shielded by 1.30 ppm in compound 251 (Figure 108). Such a shielding effect attributed to the anisotropy of the phenyl ring of the aniline is only possible if the \textit{S}_\text{N}Ar
displacement occurred at the 7-position of **344**. Similar anisotropic effects of the phenyl ring have been previously reported by Shah *et al.* in pyrolo[3,2-\(d\)]pyrimidine analogs.

![Chemical Structures](image)

**Figure 108:** Possible explanation of the phenyl ring orientation in compound **251**

The \(^1\)H NMR spectra of the \(N^7\)-H analog **252** and \(N^7\)-CH\(_3\) analog **251** in DMSO-d\(_6\) afforded additional information related to the conformational restriction in **251** (Figure 108). For **251**, the “\(N1\)-CH\(_3\)” protons appeared at \(\delta\) 3.04, whereas for **252** they were significantly deshielded at \(\delta\) 4.35 ppm. This shielding of the “\(N1\)-CH\(_3\)” protons in **251** was attributed to a diamagnetic anisotropic effect in **251** arising from the proximity of the \(N^7\)-phenyl ring as shown in Figure 108 (more favored anti-conformation for **251** on the basis of the \(N^7\)-CH\(_3\) and the \(N1\)-CH\(_3\) groups). The steric bulk of the \(N^7\)-CH\(_3\) and/or steric clash of the \(N^7\)-CH\(_3\) and the \(N1\)-CH\(_3\) groups in **251** restricts the conformation and positions the \(N^7\)-phenyl group on top of the \(N1\)-CH\(_3\) moiety resulting in the observed shielding effect, in the \(^1\)H NMR, on the \(N1\)-CH\(_3\) group in **251** as compared to that in **252**.
This shielding effect of the phenyl group on the \( N1-\text{CH}_3 \) group (\( \delta \approx 1.20 \)) was also observed for other the \( N7-\text{CH}_3 \) analogs.

**Scheme 74. Synthesis of target compound 253**

![Scheme 74](image)

**Reagents and conditions:**

a) 6-methoxy-1,2,3,4-tetrahydroquinoline, acetonitrile, reflux, 12h

Nucleophilic aromatic substitution of 344 was carried out with 6-methoxy-1,2,3,4-tetrahydroquinoline in acetonitrile to provide 1-(5-chloro-1-methyl-1\( H \)-pyrazolo[4,3-\( d \)]pyrimidin-7-yl)-6-methoxy-1,2,3,4-tetrahydroquinoline 253 in 40% yield (Scheme 74).

**Scheme 75. Synthesis of target compounds 257, 258 and 262**

![Scheme 75](image)

Reagents and conditions: a) Anilines, acetonitrile, reflux, 10-12 h
Nucleophilic aromatic substitution of 344 was done using 4-ethoxy-N-methylaniline, 4-isopropoxy-N-methylaniline, \( N \)-methyl-4-(methylthio)aniline, and \( N \)-methyl-4-(trifluoromethoxy)aniline in acetonitrile to provided 257, 258 and 262 in 36-42% yield (Scheme 75).

**Scheme 76. Synthesis of target compound 261**

\[
\text{Reagents and conditions: a) 5-methoxy-\(N\)-methylnaphthalen-2-amine, acetonitrile, reflux, 8 h}
\]

Nucleophilic aromatic substitution of 344 with 5-methoxy-\(N\)-methylnaphthalen-2-amine in acetonitrile at reflux provided 5-chloro-\(N\)-(5-methoxynaphthalen-2-yl)-\(N\),1-dimethyl-1\(H\)-pyrazolo[4,3-d]pyrimidin-7-amine 261 in 40% yield (Scheme 76).

**Scheme 77. Synthesis of target compound 263**

\[
\text{Reagents and conditions: a) Anilines, acetonitrile, reflux, 12 h.}
\]
To synthesize the final compound 263, nucleophilic aromatic substitution of 344 was done using 3-fluoro-4-methoxy-N-methylaniline in acetonitrile at reflux for 12 h to obtain 5-chloro-N-(3-fluoro-4-methoxyphenyl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine 263 46% yield (Scheme 77).

D.3. Synthesis of 6-(arylthio)pyrido[2,3-d]pyrimidine-2,4-diamines as selective pjDHFR.

**Scheme 78: Attempted synthesis of 348**

Reagents and conditions: a) Acetic acid, NBS, CH₃CN, reflux, overnight; b) Benzenethiol 347, Cs₂CO₃, ACN, MW, 80 °C, 12 h.

The initial approach for the synthesis of 282 (Scheme 78) was bromination of 2-aminonicocinonitrile 345 via NBS in acetonitrile at reflux overnight.¹²¹ The next step was to append this key intermediate 346 with various arylthiols (benzenethiol 347 for 348). García *et al.*⁴⁰⁹ reported a reaction of aryl thiol and the corresponding halo arylpyridine with Cs₂CO₃ as a base under microwave conditions. The structure of 346 was confirmed by investigating the coupling constants (*J*) of the protons [¹H NMR (500 MHz, DMSO-*d₆*) δ 8.22 (d, *J* = 2.6 Hz, 1H), 8.07 (d, *J* = 2.6 Hz, 1H), 7.12 (s, 2H, exch., 2-NH₂).] The coupling constants match the characteristic *J* constants for meta coupling between aromatic protons.⁴¹⁰ The reaction did not yield any product after 12 h at 80 °C. At higher temperature (100 °C) debromination occurs and presence
of 345 was observed from both TLC and $^1$H NMR [$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.20 (dd, $J$ = 4.8, 2.0 Hz, 1H), 7.86 (dd, $J$ = 7.6, 2.0 Hz, 1H), 6.90 (s, 2H, exch., 2-NH$_2$), 6.64 (dd, $J$ = 7.6, 4.8 Hz, 1H).] The reason for the failure of the reaction could be the low nucleophilicity of 346 as the 5-bromo position is para to the amino group which might feed electrons and decrease the electrophilicity of 346. Protection of 2-amine group of 346 with pivalic anhydride was carried out to improve the electrophilicity of 346 and then the procedure of García et al.\textsuperscript{409} was followed to synthesize 348. This attempt was unsuccessful as well.

**Scheme 79: Attempted synthesis of 282**

Reagents and conditions: a) guanidine hydrochloride, NaOMe, MeOH, reflux, 12h; b) pivalic anhydride, reflux, 4h; c) acetic acid, NBS, CH$_3$CN, reflux, overnight; d) benzenethiol, Cs$_2$CO$_3$, ACN, MW, 80 °C, 12 h; e) 1N NaOH, reflux 1 h.

Next, an alternative method was devised to synthesize 282 (Scheme 79), following a published procedure.\textsuperscript{411} Cyclization with guanidine hydrochloride with 2-aminonicotinonitrile 345 in refluxing methanol, afforded the 2,4-diaminopyrido[2,3-$d$]pyrimidines 349, which was protected using pivalic anhydride to afford $N,N'$-(pyrido[2,3-$d$]pyrimidine-2,4-diyl)bis(2,2-dimethylpropanamide) 350.\textsuperscript{411} Following Scheme 78, 350 was attempted to brominate to generate
Unfortunately, the brominating reaction did not occur. The probable reason for this failure of the brominating reaction could be bulk at the 2- and 4-positions which hinder the access bromine at 6-position. Failure of the above method led to the exploration of an alternative strategy that involved a coupling reaction to give 348 with the appropriate arylthiols using the “Ullmann coupling reaction”.

The Ullmann reaction or Ullmann coupling is a coupling reaction between aryl halides, and aromatic thiols catalyzed by copper. In Scheme 80, Table 27, various Ullman coupling conditions were attempted to synthesize 348, using 2-amino-5-bromonicotinonitrile (346) and benzenethiol 347 with and/or without copper iodide. The classical Ullmann reaction requires harsh reaction conditions. Thus, all the reactions attempted in Table 27 were at high temperatures (80-100°C) and in a high boiling point solvent (DMF).

In the first case (Table 27), K₂CO₃ was used according to the published procedure of Ullman coupling. Unfortunately, with K₂CO₃, the coupling of 346 and 347 was unsuccessful. Uyeda et al. used various bases for example, Cs₂CO₃, NaOtBu, and KOrBu along with copper iodide. Unfortunately, copper catalyzed reactions with different bases failed to afford 348 (Table 27). Next, various ligands like 2,2’-bipyridine, 1,10-phenanthroline, L-proline, and 2-picolinic acid were used along with copper iodide in DMF as solvent using a time range of 12-24 h. (Table 27). While monitoring the reaction via TLC, at higher temperature (100 °C) debrominated product 345 was observed from both TLC and ¹H NMR [¹H NMR (400 MHz, DMSO-d₆) δ 8.20 (dd, J = 4.8, 2.0 Hz, 1H), 7.86 (dd, J = 7.6, 2.0 Hz, 1H), 6.90 (s, 2H, exch., 2-NH₂), 6.64 (dd, J = 7.6, 4.8 Hz, 1H).]
Scheme 80: Ullmann coupling reaction: optimization to generate 348

Table: 27 Ullmann coupling: optimization of reaction conditions to generate 348 in DMF.

<table>
<thead>
<tr>
<th>Attempt no</th>
<th>Base</th>
<th>Cu source</th>
<th>Ligand</th>
<th>Temperature (°C)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>K₂CO₃</td>
<td>-</td>
<td>-</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>2.</td>
<td>K₂CO₃</td>
<td>CuI</td>
<td>-</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>3.</td>
<td>Cs₂CO₃</td>
<td>CuI</td>
<td>-</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>4.</td>
<td>NaOB₆-t</td>
<td>CuI</td>
<td>-</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>5.</td>
<td>KOtBu</td>
<td>CuI</td>
<td>-</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>6.</td>
<td>Cs₂CO₃</td>
<td>CuI</td>
<td>2,2'-Bipyridine</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>7.</td>
<td>Cs₂CO₃</td>
<td>CuI</td>
<td>1,10-Phenanthroline</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>8.</td>
<td>Cs₂CO₃</td>
<td>CuI</td>
<td>L-proline</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
<tr>
<td>9.</td>
<td>Cs₂CO₃</td>
<td>CuI</td>
<td>2-Picolinic acid</td>
<td>80, 100</td>
<td>No reaction</td>
</tr>
</tbody>
</table>
Scheme 81: Attempted synthesis of 348 and 282

Copper(I) iodide/ligand-catalyzed arylation of thiophenols:

Reagents and conditions: a) NIS, CH$_3$CN, 80 °C, overnight; b) Table 28 conditions; c) Chloroformamidine HCl, DMSO$_2$, 140 °C, 2h.

Table 28: Optimization of reaction conditions to generate 348 with CuI in DMF.

<table>
<thead>
<tr>
<th></th>
<th>Base</th>
<th>Ligand</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Reaction yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cs$_2$CO$_3$</td>
<td>2,2'-Bipyridine</td>
<td>100</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>Cs$_2$CO$_3$</td>
<td>1,10-Phenanthroline</td>
<td>100</td>
<td>12</td>
<td>45</td>
</tr>
<tr>
<td>3.</td>
<td>Cs$_2$CO$_3$</td>
<td>L-proline</td>
<td>100</td>
<td>12</td>
<td>52</td>
</tr>
<tr>
<td>4.</td>
<td>Cs$_2$CO$_3$</td>
<td>2-Picolinic acid</td>
<td>100</td>
<td>12</td>
<td>65</td>
</tr>
</tbody>
</table>

As at temperature 100 °C debromination of some of 346 was observed in Scheme 80, thus, starting material 346 with aryl bromine at 5-position was replaced with aryl iodide (353). Aryl iodide is often more successful than aryl bromide in many cases of Ullman coupling reaction and the reaction could be conducted at lower temperatures. Method previously described in
Scheme 78, was utilized to synthesize 2-amino-5-iodonicotinonitrile 353, where, N-iodosuccinamide (NIS) was treated portion wise with 2-aminonicotinonitrile 345 in acetonitrile at 80 °C. The structure of 353 was confirmed by investigating the coupling constants (J) of the protons [1H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.35 (d, \(J = 2.3\) Hz, 1H), 8.19 (d, \(J = 2.3\) Hz, 1H), 7.11 (s, 2H, exch., 2-NH\(_2\))]. The coupling constants match the characteristic \(J\) constants for meta coupling between aromatic protons. Several ligands and Cs\(_2\)CO\(_3\) as a base were used (Table 28) which gave success with the Ullmann coupling reaction to generate 348 at different yields (40-65%). The best yield 65% was observed in DMF at 100 °C for 12 h with ligand 2-picolinic acid and the base Cs\(_2\)CO\(_3\).

**Scheme 82:** Synthesis of the target compounds 283-289

![Scheme 82](image)

Reagents and conditions: a) Arylthiols, Cs\(_2\)CO\(_3\), CuI, picolinic acid, DMF, 100 °C, 12-24 h; c) Chloroformamidine HCl, DMSO\(_2\), 140 °C, 2-4 h

To synthesize the target compounds 283-289, (Scheme 82) various arylthiols were treated with Cs\(_2\)CO\(_3\) in a solution of DMF, followed by the addition of 2-amino-5-iodonicotinonitrile 353, CuI and 2-picolinic acid\(^{420,421}\). The mixture was stirred at 100 °C for 12-24 h to give 354-360. Intermediates 354-360 without further purification were cyclized to the target compounds 283-289 with the treatment of 354-360 with chloroformamidine HCl in DMSO\(_2\), at 140 °C for 2-4 h to afford the final compounds 283-289 in 36-64% yields.
D.4. Folate receptors (FRs) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one carbon (1C) metabolism inhibitors

D.4.1. Synthesis of N1-substituted 5-amino-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-ones

Scheme 83. Synthesis of the intermediate compounds 368-369.

Reagents and conditions: a) PdCl₂, Ph₃P, TEA, CuI, ACN, μW, 100 °C, 1 h; b) H₂, Pd/C, MeOH, rt, 12 h; c) TEA, MsCl, DCM, 0 °C, 3 h; d) NaI, acetone, reflux, 12 h.

Synthesis of the intermediate compounds 368-369 (Scheme 83) started with a palladium-catalyzed Sonogashira coupling of methyl 4-iodobenzoate 361 with the appropriate alkynes 362-363 to afford 364-365 in 84-86% yield. Catalytic hydrogenation of 364-365 afforded the saturated alcohols 366-367 in 81-83% yield.²¹¹ The alcohols 366-367 were converted to the corresponding mesylate derivatives using mesyl chloride and triethylamine at 0 °C.²²³ The mesylate derivatives were not purified and, after workup, were converted to the corresponding iodides 368-369 using the Finkelstein reaction in 69-71% yield.²²⁴
**Scheme 84.** Synthesis of the intermediate compounds 370 and 371.

![Scheme 84](image)

Reagents and conditions: a) Methyl 4-nitro-1H-pyrazole-5-carboxylate 374, DMF, K₂CO₃, r.t., 12 h.

A literature search revealed that there were no reported synthetic procedures for the intermediates 370 and 371 (Scheme 84). The synthetic method of alkylation utilized in this study was sparked by the reported synthesis of a new series of 7-aminopyrazolo[4,3-d]pyrimidine derivatives from the Colotta group.⁴⁰⁶ To a solution of DMF, methyl 4-nitro-1H-pyrazole-5-carboxylate 374 was added, followed by addition of K₂CO₃ and 4-atom chain iodide intermediate 369 at room temperature (Scheme 84). After 4 h, the reaction was not complete and starting material remained (TLC). Longer reaction time (12 h) resulted in complete consumption of the 4-nitro-1H-pyrazole-5-carboxylate. Initially, the 4-atom chain intermediates 370 and 371 were inseparable on TLC as well as column chromatography as both regioisomers had close polarity and Rf values. Repeated column chromatography (5X) was attempted to isolate intermediate 370 from 371. Finally, methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1H-pyrazole-5-carboxylate 370 and methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1H-pyrazole-3-carboxylate 371 were isolated in 32 and 64% yield, respectively.
Intermediates 370 and 371 were characterized by $^1$H NMR spectroscopy. The $^1$H NMR spectrum displayed a distinguishable peak for the regioisomers 370 and 371. For the analog 370 the H3 proton appears at $\delta$ 8.41 and for the analog 371 the H5 proton appears at $\delta$ 9.02 (Figure 109). Additionally, X-ray crystallography enabled the identification of the molecular structure of the regioisomers 371 (Figure 110).
Scheme 85. Synthesis of intermediate compounds 372 and 373

Table 29: Optimization of reaction in DMF with various bases, temperature and time range of 4-24h.

<table>
<thead>
<tr>
<th>Bases</th>
<th>Temp (°C)</th>
<th>Status of reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂CO₃</td>
<td>rt</td>
<td>Partial conversion: Mixture of 372, 373 and 374 were observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Reaction incomplete: Mixture of 368, 372, 373 and 374 were observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td>Cs₂CO₃</td>
<td>rt</td>
<td>Partial conversion: Mixture of 372, 373 and 374 were observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Reaction incomplete: Mixture of 368, 372, 373 and 374 were observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>Multiple products observed in TLC.</td>
</tr>
<tr>
<td>NaH</td>
<td>rt</td>
<td>Major spot was 373 (TLC).</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Major spot was 373 (TLC).</td>
</tr>
</tbody>
</table>
Following Scheme 84, to a solution of DMF, methyl 4-nitro-1\textit{H}-pyrazole-5-carboxylate 374 was added K$_2$CO$_3$ and the 3-atom chain iodide intermediate 368 at room temperature. With this base, unfortunately, the intermediate 372 was inseparable from the intermediate 373, even after repeated column chromatography (5X) separation attempts, the separation was unsuccessful. Several different bases and temperature conditions (Table 29) were attempted to improve the yield of 372 and thus enable a separation of 372 from 373. DMF was selected as the solvent and the TLC monitoring was carried out every hour over a 4-24 h reaction time range.

The reactions were attempted in different bases, for example, K$_2$CO$_3$, Cs$_2$CO$_3$, and NaH (Table 29). Different temperatures; 0 °C, room temperature, 50 °C, 80 °C, and 100 °C were used with bases K$_2$CO$_3$ and Cs$_2$CO$_3$. For NaH two temperatures were used: room temperature and 50 °C. The base NaH yielded 373 as a major product. As Cs$_2$CO$_3$ has a relatively high solubility in DMF and appears to act as a good base,\textsuperscript{425} it was chosen as the base at room temperature in a solution of DMF and methyl 4-nitro-1\textit{H}-pyrazole-5-carboxylate 374. To this stirred mixture was added 368 under anhydrous conditions at room temperature. This afforded the 3-atom chain methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-1\textit{H}-pyrazole-5-carboxylate 372 and methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-1\textit{H}-pyrazole-3-carboxylate 373, in 25 and 70% yields, respectively.

Table 30: Solvent effect on the reaction on Scheme 85 (Reaction conditions and reagents: Cs$_2$CO$_3$, r.t., 12h).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Intermediates</th>
<th>Yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>372</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>70</td>
</tr>
<tr>
<td>Acetone</td>
<td>372</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>70</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>372</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>66</td>
</tr>
</tbody>
</table>
Since DMF requires high temperature for preparing a plug with silica for column chromatography, various solvents other than DMF were used for example acetone and acetonitrile. To further improve the yield of 372, the reaction in Scheme 85 was optimized with different solvents: acetone and acetonitrile (Table 30). Intermediates 372 and 373 were separated successfully based on the slightly improved yield (35%) of 372 and 66% yield of 373 via column chromatography.

**Scheme 86:** Synthesis of target compound 298.

Reagents and conditions: a) 10% Pd/C, H2, 45 psi, 30 mins; b) 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea, MeOH, CH3COOH, r.t., 16 h; c) NaOMe, MeOH, rt, 12 h; d) 1N NaOH, 50 °C, 3 h; e) 2-chloro-4,6-dimethoxy-1,3,5-triazine, NMM, diethyl L-glutamate; f) 1N NaOH, r.t., 1 h.

Intermediates 372 and 370 were directly subjected to catalytic reduction426 to afford 375 and 376, respectively (Scheme 86). Condensation of 375-376 with, 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea with acetic acid as catalyst and MeOH yielded the cyclized products 377-
respectively in 40-48% yield over two steps. The hydrolysis of the protecting group was carried out in 378 in situ with aqueous sodium hydroxide at 50 °C to afford the 2-amino-4-oxo-pyrazolo[4,3-d]pyrimidine intermediates followed by conversion of the free acids to the corresponding L-glutamic acid diethyl ester 380. Peptide coupling with L-glutamic acid diethyl ester hydrochloride using 2-chloro-4,6-dimethoxy-1,3,5-triazine followed by hydrolysis with aqueous 1N NaOH at room temperature, and acidification with 1 N HCl, in the cold, afforded the target compound 298. Compounds 379 and 297 were not characterized and requires further characterization.

D.4.2. Synthesis of N2-substituted 5-amino- 2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-ones

Scheme 87: Synthesis of target compounds 301-302

Reagents and conditions: a) 10% Pd/C, H2, 45 psi, 2 h; b) 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea, MeOH, CH3COOH, r.t., 16 h; c) NaOMe, MeOH, rt, 12 h; d) 1N NaOH, 50 °C, 6 h; e) 2-chloro-4,6-dimethoxy-1,3,5-triazine, NMM, diethyl L-glutamate; f) 1N NaOH, r.t., 2 h.
Intermediates 373 and 371 (Scheme 87) were directly subjected to catalytic reduction\textsuperscript{426} to generate the 2-amino derivatives 381-382. Condensation of 381-382 with 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea with acetic acid and MeOH gave the cyclized products 383-384 in 42-49% over two steps. The hydrolysis\textsuperscript{427} of the protecting groups in 383-384 were carried out in situ with aqueous sodium hydroxide at 50 °C followed by conversion of free acids to the corresponding L-glutamic acid diethyl esters via peptide coupling reaction with L-glutamic acid diethyl ester hydrochloride using 2-chloro-4,6-dimethoxy-1,3,5-triazine\textsuperscript{393} followed by chromatographic purification to afford the coupled products 385-386 in 62-64% yields. Hydrolysis\textsuperscript{393} of 385-386 with aqueous 1N NaOH at room temperature, followed by acidification with 1 N HCl, in the cold, afforded target compounds 301-302 in 58-62% yields.

**D.4.3. Synthesis of 6-substituted 2-amino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-ones**

From a retrosynthetic point of view (Scheme 88), two general strategies were envisioned for the synthesis of target compound (4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)propoxy)-2-fluorobenzoyl)-L-glutamic acid 305. From the first strategy (Route A) it was anticipated that the key intermediate methyl 4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)propoxy)-2-fluorobenzoate 389 could be synthesized via a nucleophilic displacement of 391 with 390. Intermediate 391 could be synthesized from 392 which in turn could be obtained by cyclocondensation of 2,6-diaminopyrimidin-4(3H)-one 393 with ethyl 5-chloro-4-oxopentanoate 394.
Scheme 88. Retro synthetic route A and B for 305.

From the retrosynthetic analysis depicted in route B, (Scheme 88), the desired key intermediate methyl 4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)ethoxy)-2-fluorobenzoate 389 could also be synthesized from 2,6-dimethyl-4-chloro-furo[2,3-d]pyrimidine 392 via a coupling reaction of 392 with different halogen containing fluoro benzoates 395a-395b.
**Scheme 89:** Synthesis of intermediate 389 for target compound 305.

Reagents and conditions: a) NaOAc, H₂O, reflux, 16 h; b) LiEt₃BH, THF, 0 °C, 1h; c) Mesyl chloride or tosyl chloride, Et₃N, DCM, 0 °C, 2h; d) Table 31

**Table 31:** Reaction optimization for 389 in DMF.

<table>
<thead>
<tr>
<th>Base</th>
<th>Temp (°C)</th>
<th>Time (h)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 K₂CO₃</td>
<td>80, 100, 120, 140</td>
<td>6-24</td>
<td>No reaction</td>
</tr>
<tr>
<td>2 Cs₂CO₃</td>
<td>80, 100, 120, 140</td>
<td>6-24</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

Gangjee and coworkers⁹⁰ reported a methodology that could be adopted for the synthesis of the key intermediate 389. The 6-substituted pyrrolo[2,3-d]pyrimidine 396 (Scheme 89) was synthesized by condensation of 2,6-diaminopyrimidin-4(3H)-one 393 with ethyl 5-chloro-4-oxopentanoate 394. Reduction of the ester group of 396 to the alcohol 2-amino-6-(3-hydroxypropyl)-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one 392 and subsequent mesylation of the alcohol gave 391a. Nucleophilic displacement with methyl 2-fluoro-4-hydroxybenzoate 390 with 391a was attempted in several different bases, for example, K₂CO₃ or Cs₂CO₃, at different
temperatures (80-140 °C) and time intervals (6-24 h) (Table 31). This was unsuccessful. Tosylation of the alcohol 392 gave 391b which was subjected to nucleophilic displacement with methyl 2-fluoro-4-hydroxybenzoate 390 with K$_2$CO$_3$ or Cs$_2$CO$_3$, at different temperatures. It was anticipated that increasing the electrophilicity with a tosyl group in 391b would improve the nucleophilic displacement with 390. Unfortunately, all attempts at this nucleophilic displacement using a variety of reaction conditions with different bases, electrophiles, time and temperature variations were unsuccessful.

**Scheme 90:** Synthesis of intermediate 389 for target compound 305.

![Synthesis scheme](image)

a) CuI, 1, 10-phelanthroline, DMF, Cs$_2$CO$_3$, 110-150 °C

**Table 32:** Attempted reaction conditions for the synthesis of intermediate 389 in DMF at 110-150 °C for 12-24 h.

<table>
<thead>
<tr>
<th></th>
<th>Mol% CuI</th>
<th>Mol% 1,10-phelanthroline</th>
<th>Reaction status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>20</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>20</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>30</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

The failure of the previous strategy prompted the investigation of an alternate method. Buchwald and coworkers$^{418}$ reported the coupling of aryl halides and aliphatic alcohols with
catalytic amount of copper iodide and 1,10-phenanthroline as the ligand (Table 32). In Scheme 90, coupling of 392 with 395a and 395b with different mol% of copper iodide and 1,10-phenanthroline were attempted. However, unlike Buchwald and coworkers\textsuperscript{418} reported procedures the coupling with various halides did not afford the corresponding key intermediate 389 but gave instead the starting materials.

**Scheme 91.** Retro synthetic scheme for target 305 by route C.

![Scheme 91](image)

Failure of the above method led the exploration of a new alternative retrosynthetic strategy (Scheme 91, Route C) that involved the diazomethane reaction for the synthesis of key intermediate 389. From route C, it was anticipated that the nucleophilic substitution reaction of 390 with 399 would afford the carboxylic acid 398. Carboxylic acid 398 can be converted to the acyl chloride using oxalyl chloride followed by reaction with diazomethane to afford the α-diazoketone compound. The α-diazoketo compound can then be converted to the α-bromoketone
using HBr. Cyclo-condensation of 397 with 2,6-diamino-3H-pyrimidin-4-one, 393, hydrolysis, L-glutamate peptide coupling, and saponification would afford the desired compound 305.

**Scheme 92:** Synthesis of target compound 305.

Reagents and conditions: a) Cs₂CO₃, TBAI, DMF, 2h, 100 °C; b) CF₃COOH, DCM; r.t., 2 h; c) Oxalyl chloride, DCM, reflux, 2.5 h; d) Diazomethane, (CH₃CH₂)₂O, r.t., 1.5 h; e) 48% HBr in water, 80 °C, 2 h; f) 2,6-diaminopyrimidin-4(3H)-one, DMF, r.t., 3 days; g) 1N NaOH, MeOH, rt, 2 h; h) 2-chloro-4,6-dimethoxy-1,3,5-triazine, N-methylmorpholine, DMF, rt, 12 h; i) 1N NaOH, MeOH, rt, 1 h.
The synthesis of the key intermediate 389 was accomplished as indicated in Scheme 92. Protected carboxylic acid 400 was obtained via a nucleophilic substitution of alkyl bromide 399 with compound 390. Deprotection of the tert-butyl ester with trifluoroacetic acid yielded 398 in 88% yield. The carboxylic acid 398 was then converted to the acid chloride 401, and immediately reacted with diazomethane followed by 48% HBr in water to give the corresponding α-bromomethyl ketone 403. Condensation of 2,6-diamino-3H-pyrimidin-4-one 393 with 403 in DMF at room temperature for 3 days afforded the 2-amino-4-oxo-6-substituted-pyrrolo[2,3-d]pyrimidine 389 in 21% yield over three steps. Hydrolysis of 389 gave the corresponding free acid 387. Subsequent coupling with L-glutamate 388, using 2-chloro-4,6-dimethoxy-1,3,5-triazine as the activating agent afforded the diester 404. Hydrolysis of 404 with aqueous 1N NaOH at room temperature, followed by acidification with 1 N HCl, in the cold, gave the target compound 305 in 55% yield.
V. EXPERIMENTAL

Rotary evaporator at reduced pressure was used to carry out all evaporations. Analytical samples were dried in vacuo in a CHEM-DRY drying apparatus over P₂O₅ at 50 °C. Melting points were determined either using a MEL-TEMP, II melting point apparatus with FLUKE 51 K/J electronic thermometer or using an MPA100 OptiMelt automated melting point system and are uncorrected. Thin-layer chromatography (TLC) was performed on Whatman® PE SIL G/UV254 flexible silica gel plates or Sorbtech Silica G TLC plates of thickness 200 μm w/UV254 and the spots were visualized under 254 and 365 nm ultraviolet illumination. Proportions of solvents used for TLC are by volume. All analytical samples were homogeneous on TLC in at least two different solvent systems. Flash chromatography was carried out on the CombiFlash® Rf 200 (Teledyne ISCO) automated flash chromatography system with pre-packed RediSep® Rf normal-phase flash columns (230 to 400 meshes) of diverse sizes were used. Column chromatography was performed on the silica gel (70 to 230 meshes, Fisher Scientific) column. The amount (weight) of silica gel for column chromatography was in the range of 50-100 times the amount (weight) of the crude compounds being separated. Nuclear magnetic resonance spectra for proton (¹H NMR) were recorded on the Bruker Avance II 400 (400 MHz) or Bruker Avance II 500 (500 MHz) NMR Spectrometer with TopSpin processing software. Acquired FIDs were analyzed using MestReC 3.2 or MestReNova 9.0. The chemical shift values (δ) are expressed in, (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet; td, triplet of doublet; dt, doublet of triplet; quin, quintet; exch., exchangeable using D₂O. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within ± 0.4% of the
calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented despite 24 to 48 hours of drying in vacuo and were confirmed where possible by their presence in the $^1$H NMR spectra. Mass spectrum data were acquired on an Advion expression® CMS system using the ESI probe. HPLC data were acquired on ThermoFisher Scientific UltiMate™ 3000 HPLC system.

4-chloro-2-methylthieno[2,3-d]pyrimidine (308)

Commercially available ethyl 2-aminothiophene-3-carboxylate 306 (5.0 g, 29.20 mmol) was dissolved in 25 mL of acetonitrile and hydrogen chloride gas was bubbled through the solution for 30 mins. The mixture was stirred at room temperature for overnight. The solvent was evaporated under reduced pressure. The residue was dissolved in 10 mL distilled water and treated with ammonia in water solution which generated light yellow precipitate. The precipitate was collected by filtration to afford 2.18 g (13.14 mmol, 45%) of 2-methylthieno[2,3-d]pyrimidin-4(3H)-one 307 as a light yellow solid. TLC $R_f = 0.21$ (CHCl$_3$: MeOH, 20:1); mp, 208-209 °C (lit.$^{428}$ 210 °C). Without further characterization of 307, chlorination of 307 (2.0 g, 12.03 mmol) was carried out using of POCl$_3$ (1.13 mL, 12.03 mmol) and pyridine (0.96 mL, 12.03 mmol) and refluxed for 12 hours in toluene. The solvent was evaporated and cooled in an ice bath followed by neutralization with ammonia in water solution. Yellow precipitate was formed and was collected by filtration to afford 308 as yellow solid (1.38 g, 62% yield). TLC $R_f = 0.40$ (Hexane: EtOAc, 10:1); mp, 91.6-92.5 °C (lit.$^{428}$ 93 °C); $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.01 (d, $J = 6.0$ Hz, 1H, Ar), 7.51 (d, $J = 6.0$ Hz, 1H, Ar), 2.71 (s, 3H, 2-CH$_3$). The $^1$H-NMR matched the $^1$H-NMR reported in the literature.$^{428}$ This compound was used for the next reaction without further characterization.
**General method for synthesis of 182-185 and 189**

Intermediate **308** (0.20 g, 1.08 mmol) and equivalent anilines were dissolved in 10-12 ml isopropanol and heated in a microwave reactor at 120 °C for 6-10 hours. The solvent was evaporated in vacuo, silica gel was added to make a plug and flash chromatography was carried out using ethyl acetate-hexane as eluent. The fractions containing the required compound were evaporated under reduced pressure to afford the target compounds.

**N-(4-methoxyphenyl)-2-methylthieno[2,3-d]pyrimidin-4-amine (183)**

Compound **308** (200 mg, 1.08 mmol) was dissolved in isopropanol (10 mL) followed by addition of 1 drop of HCl and reacted with 4-methoxyaniline (133.40 mg, 1.08 mmol) at microwave condition at 120 °C for 6 h. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford intermediate **183** as a white solid (182.22 mg, 62% yield). TLC Rf = 0.27 (CHCl₃: MeOH, 20:1); mp, 138-139.6 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 9.55 (s, 1H, exch., N⁴-H), 7.85 (d, J = 8.8 Hz, 2H, Ar), 7.81 (d, J = 6.0 Hz, 1H, Ar), 7.60 (d, J = 6.0 Hz, 1H, Ar), 7.30 (d, J = 8.8 Hz, 2H, Ar), 3.80 (s, 3H, -OCH₃), 2.48 (s, 3H, 2-CH₃). Anal. Calcd. for C₁₄H₁₃N₃OS 0.26 (CH₃)₂CHOH. 0.35 HCl: C, 59.21; H, 5.19; N, 14.00; S, 10.69. Found: C, 59.25; H, 4.95; N, 14.04; S, 10.45.

**N-(4-methoxyphenyl)-N,2-dimethylthieno[2,3-d]pyrimidin-4-amine (182):**

A solution of **183** (150 mg, 0.552 mmol) in DMF (10 ml) followed by addition of portion wise sodium hydride (60 % dispersion in mineral oil) (22.11 mg, 0.552 mmol). The reaction was stirred at room temperature for 10 mins and then iodomethane (0.04 mL, 0.663 mmol) dissolved in 5 mL
of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 182 as a white solid (123 mg, 78% yield). TLC $R_f = 0.44$ (Hexane: EtOAc, 3:1); mp, 155.3-157 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.31 (d, $J = 8.8$ Hz, 2H, Ar), 7.15 (d, $J = 6.2$ Hz, 1H, Ar), 7.07 (d, $J = 8.8$ Hz, 2H, Ar), 5.45 (d, $J = 6.2$ Hz, 1H, Ar), 3.83 (s, 3H, -OCH$_3$), 3.49 (s, 3H, -N$_4$CH$_3$), 2.53 (s, 3H, -CH$_3$). Anal. Calcd. for C$_{15}$H$_{15}$N$_3$OS: C, 63.13; H, 5.29; N, 14.73; S, 11.23. Found: C, 63.43; H, 5.40; N, 14.83; S, 11.15.

**N,2-dimethyl-N-(4-(methylthio) phenyl)thieno[2,3-d]pyrimidin-4-amine (184):**

Compound 308 (200 mg, 1.08 mmol) was dissolved in isopropanol (10 mL) followed by addition of 1 drop of HCl and reacted with 4-(methylthio)aniline (180.96 mg, 1.30 mmol) at microwave condition at 120 °C for 8 h to afford 184a. Crude 184a (150 mg, 0.521 mmol) without further characterization was dissolved in DMF (10 ml) followed by portion wise addition of sodium hydride (60% dispersed in mineral oil) (20.87 mg, 0.521 mmol). The reaction was stirred at room temperature for 10 mins and then iodomethane (0.039 mL, 0.626 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 184 as a white solid (81 mg, 52% yield in two steps). TLC $R_f = 0.5$ (Hexane: EtOAc, 3:1); mp, 153.2-155 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.38 (d, $J = 8.7$ Hz, 2H, Ar), 7.32 (d, $J = 8.7$ Hz, 2H, Ar), 7.21 (d, $J = 6.2$ Hz, 1H, Ar), 5.57 (d, $J = 6.1$ Hz, 1H, Ar), 3.50 (s, 3H, -N$_4$CH$_3$), 2.54 (s, 3H, -CH$_3$), 2.53 (s, 3H, -CH$_3$). Anal. Calcd. for
C₁₅H₁₅N₃S₂: C, 59.77; H, 5.02; N, 13.94; S, 21.28. Found: C, 60.00; H, 4.97; N, 13.93; S, 21.01.

4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methylthieno[2,3-d]pyrimidine (185)

To a solution of 308 (200 mg, 1.08 mmol) in isopropanol (10 mL), 1 drop of HCl was added, followed by addition of 6-methoxy-1,2,3,4-tetrahydroquinoline (194.48 mg, 1.19 mmol) at microwave condition at 120 °C for 6 h. The reaction mixture was cooled to r.t., and silica gel (500 mg) was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using hexane and ethyl acetate in a ratio of 10:1 to give an off-white solid as 185 in 76% yield. (256 mg). TLC Rₙ = 0.38 (Hexane: EtOAC, 3:1); mp 195-196.8 °C; ¹H NMR (400 MHz, DMSO-δ₆) δ 7.35 (d, J = 6.1 Hz, 1H, Ar), 6.91 (d, J = 8.8 Hz, 1H, Ar), 6.89 (d, J = 3.0 Hz, 1H, Ar), 6.70 (dd, J = 8.8 Hz, J = 3.0 Hz, 1H, Ar), 6.27 (d, J = 6.2 Hz, 1H, Ar), 3.97 (t, J = 6.5 Hz, 2H, -CH₂-), 3.76 (s, 3H, -OCH₃), 2.75 (t, J = 6.5 Hz, 2H, -CH₂-), 2.54 (s, 3H, -CH₂-), 1.99-1.90 (m, 2H, -CH₂-). Anal. Calcd. for C₁₇H₁₇N₃OS: C, 65.57; H, 5.50; N, 13.49; S, 10.29. Found: C, 65.62; H, 5.51; N, 13.26; S, 10.19.

N-(5-methoxynaphthalen-2-yl)-N₂-dimethylthieno[2,3-d]pyrimidin-4-amine (189)

To a solution of 308 (200 mg, 1.08 mmol) in isopropanol (10 mL), 1 drop of HCl was added, followed by addition of 6-aminonaphthalen-1-ol (206.92 mg, 1.30 mmol) at microwave condition at 120 °C for 8 h. The reaction mixture was cooled to rt, silica gel (500 mg) was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using hexane and ethyl acetate (10:1) to give off-white solid. TLC Rₙ = 0.28 (CHCl₃: MeOH, 20:1). Crude (120 mg, 0.390 mmol) without further characterization was dissolved in DMF (10 ml) followed by portion wise addition of sodium hydride (60%
dispersed in mineral oil) (15.61 mg, 0.390 mmol). The reaction was stirred at room temperature for 10 mins and then iodomethane (0.03 mL, 0.468 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 189 as a white solid (104 mg, 48% yield). TLC \( R_f = 0.34 \) (Hexane: EtOAc, 3:1); mp, 206.2-207.8 °C; \(^1\)H NMR (400 MHz) (Me2SO-\(d_6\)) \( \delta \) 8.26 (d, \( J = 8.8 \) Hz, 1 H, Ar), 7.97 (d, \( J = 2.3 \) Hz, 1 H, Ar), 7.80 (d, \( J = 5.5 \) Hz, 1 H, Ar), 7.55 (dd, \( J = 8.8, 2.3 \) Hz, 1 H, Ar), 7.51 – 7.48 (m, 2 H, Ar), 7.28 (d, \( J = 5.5 \) Hz, 1 H, Ar), 7.10 – 7.02 (m, 1 H, Ar), 3.99 (s, 3 H, -OCH\(_3\)), 3.61 (s, 3 H, -N\(^4\)CH\(_3\)), 2.52 (s, 3H, 2-CH\(_3\)). Anal. Calcd. for C\(_{19}\)H\(_{17}\)N\(_3\)OS: C, 68.03; H, 5.11; N, 12.52; S, 9.56. Found: C, 68.25; H, 5.25; N, 12.41; S, 9.52.

\(^N\)-(4-methoxyphenyl)-\(^N\)-methylthieno[2,3-\(d\)]pyrimidin-4-amine (196)

Commercially available 4-chlorothieno[2,3-\(d\)]pyrimidine 309 (300 mg, 1.11 mmol) in isopropanol (10 mL) was treated with 1 drop of HCl and 4-methoxy-\(^N\)-methylaniline (183.08 mg, 1.33 mmol) at microwave condition at 120 °C for 6 hours. The reaction was cooled to room temperature and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 196 in 60% as yellow solid (247mg). TLC \( R_f = 0.41 \) (Hexane: EtOAc, 1:1); mp, 131.2-133.1 °C; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \( \delta \) 8.52 (s, 1H, Ar), 7.34 (d, \( J = 9.0 \) Hz, 2H, Ar), 7.29 (d, \( J = 6.2 \) Hz, 1H, Ar), 7.08 (d, \( J = 9.0 \) Hz, 2H, Ar), 5.52 (d, \( J = 6.2 \) Hz, 1H, Ar), 3.83 (s, 3H, -OCH\(_3\)), 3.51 (s, 3H, -N\(^4\)CH\(_3\)). Anal. Calcd. for C\(_{15}\)H\(_{15}\)N\(_3\)S\(_2\)C, 59.77; H, 5.02; N, 13.94; S, 21.27. Found: C, 59.69; H, 5.00; N, 14.15; S, 21.33.
**4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)thieno[2,3-d]pyrimidine (198)**

Commericially available 4-chlorothieno[2,3-d]pyrimidine 309 (300 mg, 1.11 mmol) in isopropanol was treated with 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline (217.8 mg, 1.33 mmol) at microwave conditions at 120 °C for 8 hours. The reaction was cooled to room temperature and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 198 in 58% as yellow solid (255.7 mg). TLC $R_f$ = 0.26 (Hexane: EtOAc, 3:1); mp, 140.3-141.8 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.48 (s, 1H, Ar), 7.31 (d, $J = 8.9$ Hz, 1H, Ar), 7.29 (d, $J = 3.0$ Hz, 1H, Ar), 7.26 (d, $J = 6.1$ Hz, 1H, Ar), 7.02 (dd, $J = 8.9$ Hz, $J =3.0$ Hz, 1H, Ar), 5.62 (d, $J = 6.1$ Hz, 1H, Ar), 3.90 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 3.78 (s, 3H, -OCH$_3$), 2.71 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 1.98-1.89 (m, 2H, -CH$_2$-). Anal. Calcd. for C$_{16}$H$_{15}$N$_3$OS: C, 64.62; H, 5.08; N, 14.13; S, 10.63. Found: C, 64.61; H, 5.25; N, 14.13; S, 10.63.

**N4-(4-methoxyphenyl)-N4-methylthieno[2,3-d]pyrimidine-2,4-diamine (197)**

Methyl sulfone (15 g), commercially available ethyl 2-aminothiophene-3-carboxylate 306 (5.0 g, 29.20 mmol) and chloroformamidine hydrochloride (6.71 g, 58.41 mmol) were taken in a round bottom flask. The reaction mixture was heated at 140 °C for 4 h. The reaction was quenched with 100 mL water, cooled in an ice bath and was basified to pH 8.0 using an aqueous NH$_4$OH solution. The precipitate was collected by filtration, dried (using Na$_2$SO$_4$) and afforded 2.93 g (60% yield) of 2-aminothieno[2,3-d]pyrimidin-4(3H)-one 310 as a brown solid. TLC $R_f$ = 0.15 (MeOH: CHCl$_3$:NH$_4$OH, 1:5:0.5). Intermediate 310 (2.8 g, 16.75 mmol), without further characterization, was added to a 250 mL round bottom flask and Piv$_2$O (34 mL, 167.48 mmol) was added. The reaction mixture was vigorously stirred at reflux condition.
After 20 min, the reactant was completely dissolved, and, after 2 hours, the product was precipitated. The crude product (2.95 g, 11.72 mmol, 70% yield) was not subjected to separation efforts and was taken to the next step without any characterization. To a 100 mL round bottom flask were added the crude 310 (2.93 g, 11.66 mmol), POCl₃ (1.09 mL, 11.66 mmol) and pyridine (0.94 mL, 11.66 mmol). The reaction mixture was heated to reflux for 10 h. The POCl₃ was evaporated, and the mixture was neutralized using an aqueous NH₄OH solution to yield a precipitate. The precipitate 311 (2.20 g, 49% yield) was collected by filtration, washed with water, dried and then dissolved in MeOH. The product N-(4-chlorothieno[2,3-d]pyrimidin-2-yl)pivalamide 311 was not subjected to separation and the precipitate crude was taken to the next step without any characterization. To a solution of 311 (300 mg, 1.11 mmol) in isopropanol (10 mL), 1 drop of HCl was added, followed by the addition of 4-methoxy-N-methylaniline (183.03 mg, 1.33 mmol) under reflux condition for 12 hours. After cooling to r.t., 1 N NaOH solution (4 mL) was added and the reaction mixture was heated to reflux for 2 hours. The reaction mixture was cooled to r.t., silica gel (500 mg) was added, and the solvent was removed under reduced pressure. Purification was performed by flash column chromatographic separation using ethyl acetate and hexane as eluent, and the fractions containing the product (TLC) were pooled, evaporated to afford 152.7 mg (48% in two steps) of 197 as a white solid. TLC $R_f = 0.25$ (Hexane: EtOAc, 1:1); mp 202.6 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.27 (d, $J = 8.9$ Hz, 2H, Ar), 7.05 (d, $J = 8.9$ Hz, 2H, Ar), 6.65 (s, $J = 6.3$ Hz, 1H, Ar), 6.28 (s, 2H, exch., 2-NH$_2$), 5.29 (s, $J = 6.3$ Hz, 1H, Ar), 3.82 (s, 3H, -OCH$_3$), 3.49 (s, 3H, -N$_4$-CH$_3$). Anal. Calcd. for C$_{14}$H$_{14}$N$_4$OS 0.06 CH$_3$(CH$_2$)$_4$CH$_3$: C, 59.17; H, 5.13; N, 19.21; S, 10.99. Found: C, 58.82; H, 5.24; N, 18.90; S, 10.86.
4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)thieno[2,3-d]pyrimidin-2-amine (199)

To a solution of 311 (300 mg, 1.11 mmol) in isopropanol (10 mL), 1 drop of HCl was added, followed by addition of 6-methoxy-1,2,3,4-tetrahydroquinoline (235.98 mg, 1.45 mmol). The reaction mixture was stirred for 8 hours at reflux condition. After cooling to rt, 1 N NaOH solution (4 mL) was added and heated to reflux for 2 hours. The reaction mixture was cooled to r.t., silica gel (500 mg) was added, and the solvent was removed under reduced pressure. Purification was performed by flash column chromatographic separation using 5% MeOH in CHCl₃ as the eluent, and the fractions containing the product (TLC) were pooled, evaporated to give a solid which was then washed with CHCl₃ to afford 139 mg (40% in two steps) of 199 as white solid. TLC Rf = 0.55 (MeOH:CHCl₃:NH₄OH, 1:10:0.5); mp, 209-210 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 6.87 (d, J = 6.1 Hz, 1H, Ar), 6.85 (d, J = 8.9 Hz, 1H, Ar), 6.83 (d, J = 2.9 Hz, 1H, Ar), 6.69 (dd, J = 8.8 Hz, J =2.9 Hz, 1H, Ar), 6.38 (s, 2H, exch., 2-NH₂), 6.10 (d, J = 6.1 Hz, 1H, Ar), 3.96 (t, J = 6.6 Hz, 2H, -CH₂-), 3.75 (s, 3H, -OCH₃), 2.78 (t, J = 6.5 Hz, 2H, -CH₂-). Anal. Calcd. for C₁₆H₁₆N₄OS 0.17 CH₃OH: C, 61.11; H, 5.29; N, 17.63; S, 10.09. Found: C, 61.09; H, 5.24; N, 17.63; S, 10.10.

2-chloro-N-(4-methoxyphenyl)-N-methylthieno[2,3-d]pyrimidin-4-amine (204)

Commercially available 2,4-dichlorothieno[2,3-d]pyrimidine 312 (400 mg, 1.95 mmol) in isopropanol (10 mL) was treated with 4-methoxy-N-methylaniline (267.60 mg, 1.95 mmol) and reacted for 48 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate and hexane as eluent to afford 204 in 226.6 mg as pale-yellow solid (38% yield). TLC Rf = 0.52 (Hexane: EtOAc, 3:1); mp, 156.7-159 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.45 (d, J = 6.2 Hz, 1H, Ar), 7.15 (d, J = 8.8 Hz, 2H, Ar), 7.07 (d, J = 8.8 Hz, 2H, Ar), 6.22 (d, J
2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)thieno[2,3-d]pyrimidine (205)

Commercially available 2,4-dichlorothieno[2,3-d]pyrimidine 312 (400 mg, 1.95 mmol) in isopropanol (10 mL) was treated with 6-methoxy-1,2,3,4-tetrahydroquinoline (318.39 mg, 1.95 mmol) and treated for 48 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 205 in 226 mg as pale-yellow solid (35% yield).

TLC $R_f = 0.36$ (Hexane: EtOAc, 3:1); mp, 168-169.8 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.48 (d, $J = 6.1$ Hz, 1H, Ar), 7.06 (d, $J = 8.7$ Hz, 1H, Ar), 6.93 (d, $J = 2.9$ Hz, 1H, Ar), 6.74 (dd, $J = 8.7$ Hz, $J = 2.9$ Hz, 1H, Ar), 6.28 (d, $J = 6.1$ Hz, 1H, Ar), 3.95 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 3.78 (s, 3H, -OCH$_3$), 2.74 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 1.98 – 1.87 (m, 2H, -CH$_2$-). Anal. Calcd. for C$_{16}$H$_{14}$ClN$_3$OS 0.13 CH$_3$(CH$_2$)$_2$CH$_3$: C, 58.76; H, 4.65; Cl, 11.33; N, 12.24; S, 9.34. Found: C, 58.61; H, 4.55; Cl, 9.94; N, 12.36; S, 9.06.

4-chloro-2,5-dimethylthieno[2,3-d]pyrimidine (315a)

To a 50 mL flask were added commercially available ethyl 2-amino-4-methylthiophene-3-carboxylate 313 (5.0 g, 26.99 mmol) and acetonitrile (20 mL). The resulting mixture was stirred at room temperature to afford a clear solution. Anhydrous HCl gas was bubbled into the solution for 30 mins until there was no further precipitation and then stirred for an additional hour. The residue was dried and dissolved in 10 mL distilled water and treated with ammonia in water solution to generate precipitate. The white precipitate was collected through
filtration and washed with ether then dried over P₂O₅ to afford 3.16 g of 314 (65% yield). TLC Rf = 0.13 (Hexane: EtOAc, 3:1); mp, >250 °C (lit. 263 °C); ¹H NMR (400 MHz, DMSO-d₆) δ 12.27 (s, 1H, exch., -NH), 7.61 (s, 1H, Ar), 2.68 (s, 3H, 5-CH₃), 2.58 (s, 3H, 2-CH₃). Crude 314 (3.0 g, 16.65 mmol) without further characterization was dissolved in toluene (30 mL) followed by addition of POCl₃ (1.56 mL, 16.65 mmol), and pyridine (1.34 mL, 16.65 mmol). The resulting mixture was refluxed for 12 hours, and the solvent was removed under reduced pressure to afford a dark residue. The solvent was evaporated and neutralized with ammonia in water solution to generate light-yellow precipitate. The precipitate was collected by filtration to afford crude 315a. Silica gel column chromatography using hexane/EtOAc = 20:1 as the eluent was used to separate 315a. Fractions containing the product (TLC) were combined and evaporated to afford 315a as a light-yellow solid (1.6 g, 48% yield); TLC Rf = 0.13 (Hexane: EtOAc, 3:1); mp, 252.5-253.6 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.21 (s, 1H, Ar), 2.45 (s, 3H, 5-CH₃), 2.34 (s, 3H, 2-CH₃). The ¹H-NMR matched the ¹H-NMR reported in the literature. This compound was used for the next reaction without further characterization.

**N-(4-methoxyphenyl)-N,2,5- trimethylthieno[2,3-d]pyrimidin-4-amine (218)**

Compound 315a (300 mg, 1.51 mmol) was dissolved in isopropanol (10 mL) followed by addition of 1 drop of HCl and 4-methoxy-N-methylaniline (227.87 mg, 1.66 mmol). The resulting mixture was refluxed for 12 hours. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 218 as a white solid (280 mg, 62% yield). TLC Rf = 0.56 (Hexane: EtOAc, 3:1); mp, 236-237.8 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.02 (s, 1H, Ar), 6.94 (d, J = 8.4 Hz, 2H, Ar), 6.86 (d, J = 8.4 Hz,
2H, Ar), 3.71 (s, 3H, -OCH₃), 3.44 (s, 3H, -N₄CH₃), 2.58 (s, 3H, 2-CH₃), 1.54 (s, 3H, 5-CH₃).
Anal. Calcd. for C₁₆H₁₇N₃O₃S: C, 64.19; H, 5.72; N, 14.03; S, 10.71. Found: C, 64.35; H, 5.73; N, 13.97; S, 10.60.

4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl) -2,5-dimethylthieno[2,3-d]pyrimidine (221)
Compound 315a (200 mg, 1.01 mmol) was dissolved in isopropanol followed by addition of 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline (180.75 mg, 1.11 mmol). The reaction mixture was stirred for 6 hours at reflux condition. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using hexane and EtOAc as eluent to afford 221 as a white solid (196.5 mg, 60% yield). TLC Rf = 0.36 (Hexane: EtOAc, 10:1); mp, 245.8-247 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.22 (s, 1H, Ar), 6.90 (d, J = 8.8 Hz, 1H, Ar), 6.85 (d, J = 3.0 Hz, 1H, Ar), 6.70 (dd, J = 8.8 Hz, J =3.0 Hz, 1H, Ar), 3.96 (t, J = 6.5 Hz, 2H, -CH₂-), 3.71 (s, 3H, -OCH₃), 2.72 (t, J = 6.5 Hz, 2H, -CH₂-), 2.58 (s, 3H, 2-CH₃), 1.98-1.90 (m, 2H, -CH₂-), 1.54 (s, 3H, 5-CH₃). Anal. Calcd. for C₁₈H₁₉N₃O₃S: C, 66.43; H, 5.88; N, 12.91; S, 9.85. Found: C, 66.37; H, 5.98; N, 13.00; S, 9.68.

2-chloro-N-(4-methoxyphenyl)-N,5-dimethylthieno[2,3-d]pyrimidin-4-amine (219)
Commercially available 2,4-dichloro-5-methylthieno[2,3-d]pyrimidine 315b (300 mg, 1.37 mmol) was dissolved in isopropanol (10 mL) at room temperature followed by addition of 1 drop of HCl and 4-methoxy-N-methylaniline (187.85 mg, 1.37 mmol) and reacted for 18 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford intermediate 219 as light yellow solid (210 mg, 48% yield); TLC Rf = 0.65 (Hexane:
EtOAc, 3:1); mp, 216-217.5 °C; \( ^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.14 (s, 1H, Ar), 7.10 (d, \(J = 9.0\) Hz, 2H, Ar), 6.90 (d, \(J = 9.0\) Hz, 2H, Ar), 3.74 (s, 3H, -OCH\(_3\)), 3.46 (s, 3H, -N\(^4\)CH\(_3\)), 1.45 (s, 3H, 5-CH\(_3\)). Anal. Calcd. for C\(_{15}\)H\(_{14}\)ClN\(_3\)OS 0.16 (CH\(_3\))\(_2\)CHOH: C, 56.43; H, 4.67; Cl, 10.76; N, 12.76; S, 9.73. Found: C, 56.10; H, 4.78; Cl, 10.64; N, 12.90; S, 9.65.

**N4-(4-methoxyphenyl)-N4,5-dimethylthieno[2,3-\(d\)]pyrimidine-2,4-diamine (220)**

Commercially available 4-chloro-5-methylthieno[2,3-\(d\)]pyrimidin-2-amine 315c (250 mg, 1.14 mmol) was dissolved in isopropanol (10 mL) at r.t. followed by addition of 1 drop of HCl and 4-methoxy-N-methylaniline (156.5 mg, 1.14 mmol) for 24 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using methanol-dichloromethane as eluent to afford intermediate 220 as light yellow solid (164 mg, 45% yield); TLC \(R_f\) = 0.20 (MeOH:CH\(_2\)Cl\(_2\):NH\(_4\)OH, 1:20:0.5); mp, 209.5-211 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.02 (s, 1H, Ar), 7.10 (d, \(J = 8.9\) Hz, 2H, Ar), 6.98 (d, \(J = 8.9\) Hz, 2H, Ar), 5.96 (s, br, 2H, exch., 2-NH\(_2\)), 3.81 (s, 3H, -OCH\(_3\)), 3.42 (s, 3H, -N\(^4\)CH\(_3\)), 1.58 (s, 3H, 5-CH\(_3\)). Anal. Calcd. for C\(_{15}\)H\(_{16}\)N\(_4\)OS 0.44 CH\(_2\)Cl\(_2\): C, 54.88; H, 5.04; N, 16.58; S, 9.48. Found: C, 54.82; H, 5.36; N, 16.71; S, 9.44.

**2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)-5-methylthieno[2,3-\(d\)]pyrimidine (222)**

Commercially available 2,4-dichloro-5-methylthieno[2,3-\(d\)]pyrimidine 315b (200 mg, 0.91 mmol) was dissolved in isopropanol (10 mL) at r.t. followed by addition of 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline (149 mg, 0.91 mmol) for 18 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash
column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford intermediate 222 as yellow solid (142 mg, 45% yield). TLC $R_f = 0.47$ (Hexane: EtOAc, 3:1); mp, 220-222.8 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.25 (s, 1H, Ar), 6.81 ($J = 8.8$ Hz, 1H, Ar), 6.60 (d, $J = 2.9$ Hz, 1H, Ar), 6.53 (dd, $J = 8.8$ Hz, $J = 2.9$ Hz, 1H, Ar), 3.81 (t, $J = 6.4$ Hz, 2H, -CH$_2$-), 3.72 (s, 3H, -OCH$_3$), 2.79 (t, $J = 6.4$ Hz, 2H, -CH$_2$-), 1.74-1.80 (m, 2H, -CH$_2$-) 1.64 (s, 3H, 5-CH$_3$). Anal. Calcd. for C$_{17}$H$_{16}$ClN$_3$OS $0.29$ CH$_3$(CH$_2$)$_4$CH$_3$ 0.19 HCl: C, 59.51; H, 5.39; Cl, 11.24; N, 11.13; S, 8.49. Found: C, 59.80; H, 5.44; Cl, 11.50; N, 11.51; S, 8.50.

4-(6-methoxy-3,4-dihydroquinolin-1(2$H$)-yl)-5-methylthieno[2,3-$d$]pyrimidin-2-amine (223)

Commercially available 4-chloro-5-methylthieno[2,3-$d$]pyrimidin-2-amine 315c (220 mg, 1.0 mmol) was dissolved in isopropanol (10 mL) at r.t. followed by addition of 1 drop of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline (137.8 mg, 1.0 mmol) for 24 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using methanol-dichloromethane as eluent to afford intermediate 223 as light yellow solid (128.5 mg, 40% yield); TLC $R_f = 0.24$ (MeOH:CH$_2$Cl$_2$:NH$_4$OH, 1:20:0.5); mp, 218-220.2 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.15 (s, 1H, Ar), 6.84 ($J = 8.9$ Hz, 1H, Ar), 6.62 (d, $J = 3.0$ Hz, 1H, Ar), 6.56 (dd, $J = 8.9$ Hz, $J = 3.0$ Hz, 1H, Ar), 5.92 (s, br, 2 H, exch., 2-NH$_2$), 3.78 (t, $J = 6.3$ Hz, 2H, -CH$_2$-), 3.70 (s, 3H, -OCH$_3$), 2.76 (t, $J = 6.3$ Hz, 2H, -CH$_2$-), 1.70-1.78 (m, 2H, -CH$_2$-), 1.60 (s, 3H, 5-CH$_3$). Anal. Calcd. for C$_{17}$H$_{18}$N$_4$OS: C, 62.55; H, 5.55; N, 17.16; S, 9.82. Found: C, 62.29; H, 5.69; N, 16.88; S, 9.62.
4-chloro-2,5,6-trimethylthieno[2,3-d]pyrimidine (318)

To a 50 mL flask were added commercially available ethyl 2-amino-4,5-dimethylthiophene-3-carboxylate 316 (6.0 g, 30.11 mmol) and acetonitrile (20 mL). The resulting mixture was stirred to afford a clear solution. Anhydrous HCl gas was bubbled into the solution for 30 mins until there was no further precipitation. The mixture was stirred for an additional hour at room temperature. The residue was dried and dissolved in 10 mL distilled water and treated with ammonia in water solution to generate precipitate. The white precipitate was collected through filtration and washed with ether then dried over P₂O₅ to afford 3.80 g of yellow solid of 317 (65% yield). TLC $R_f = 0.38$ (CHCl₃: MeOH, 20:1); mp >250 °C (lit.⁴⁰⁴ 266 °C) $^1$H NMR (400 MHz, DMSO-d₆): δ 11.98 (s, 1H, exch., -NH), 2.41 (s, 3H, -CH₃), 2.36 (s, 3H, -CH₃), 2.30 (s, 3H, -CH₃). Crude 317 (3.50 g, 18.02 mmol) without further characterization was dissolved in toluene (20 mL), POCl₃ (1.68 mL, 18.02 mmol) and pyridine (1.45 mL, 18.02 mmol). The resulting mixture was refluxed for 12 hours, and the solvent was removed under reduced pressure and neutralized with ammonia in water solution to generate light-yellow precipitate. The precipitate was collected by filtration to afford crude 318. Silica gel column chromatography using hexane and EtOAc (10:1) as the eluent was used to separate 318. Fractions containing the product (TLC) were combined and evaporated to afford 318 as a light-yellow solid (1.92 g, 50% yield). TLC $R_f = 0.57$ (Hexane: EtOAc 3:1); mp 116.8-118 °C (lit.⁴²⁹ 116-117 °C) $^1$H NMR (400 MHz, DMSO-d₆): δ 2.47 (s, 3H, -CH₃), 2.38 (s, 3H, -CH₃), 2.32 (s, 3H, -CH₃). Anal. Calcd. for C₉H₉ClN₂S: C, 50.82; H, 4.26; Cl, 16.67; N, 13.17; S, 15.08. Found: C, 50.75; H, 4.56; Cl, 16.53; N, 13.19; S, 14.99.
N-(4-methoxyphenyl)-2,5,6-trimethylthieno[2,3-d]pyrimidin-4-amine (227)

In a solution of isopropanol (10 mL), 318 (300 mg, 1.41 mmol) were added 1 drop of HCl and 4-methoxyaniline (191.08 mg, 1.55 mmol) and refluxed for 12 hours. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 227 as a white solid (253 mg, 60% yield). TLC $R_f = 0.44$ (Hexane: EtOAc, 3:1); mp, 176.3-178 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.04 (s, 1H, exch., $N^4$-H), 7.58 (d, $J = 8.6$ Hz, 2H, Ar), 6.94 (d, $J = 8.6$ Hz, 2H, Ar), 3.76 (s, 3H, -OCH$_3$), 2.56 (s, 3H, 2-CH$_3$), 2.42 (s, 3H, -CH$_3$), 2.40 (s, 3H, -CH$_3$). Anal. Calcd. for C$_{16}$H$_{17}$N$_3$O: C, 64.19; H, 5.72; N, 14.03; S, 10.71. Found: C, 64.41; H, 5.91; N, 13.80; S, 10.43.

N-(4-methoxyphenyl)-N,N',2,5,6-tetramethylthieno[2,3-d]pyrimidin-4-amine (225)

In a solution of DMF (10 ml), 227 (200 mg, 0.668 mmol) was added portion wise sodium hydride (60% dispersed in mineral oil) (26.72 mg, 0.668 mmol). The reaction was stirred at room temperature for 10 mins and then iodomethane (0.5 mL, 0.808 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 225 as a light yellow solid (144.5 mg, 69% yield); TLC $R_f = 0.63$ (Hexane: EtOAc, 3:1); mp, 187-189.6 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.89 (d, $J = 9.1$ Hz, 2H, Ar), 6.84 (d, $J = 9.1$ Hz, 2H, Ar), 3.71 (s, 3H, -OCH$_3$), 3.43 (s, 3H, -N$^4$CH$_3$), 2.57 (s, 3H, 2-CH$_3$), 2.25 (s, 3H, 6-CH$_3$), 1.53 (s, 3H, 5-CH$_3$). Anal. Calcd. for C$_{15}$H$_{15}$N$_3$S$_2$C, 59.77; H, 5.02; N, 13.94; S, 21.27. Found: C, 59.69; H, 5.00; N, 14.15; S, 21.33.
2,5,6-trimethyl-N-(4-(methylthio)phenyl)thieno[2,3-d]pyrimidin-4-amine (228)

In a solution of isopropanol (10 mL), 318 (250 mg, 1.18 mmol) were added 1 drop of HCl and 4-(methylthio)aniline (0.16 mL, 1.29 mmol) and refluxed for 12 hours. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 228 as a white solid (252 mg, 68% yield). TLC Rf = 0.32 (Hexane: EtOAc, 3:1); mp, 178-179.6 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 8.12 (s, 1H, exch., -N⁴H), 7.62 (d, J = 8.6 Hz, 2H, Ar), 7.22 (d, J = 8.6 Hz, 2H, Ar), 2.45 (s, 3H, -CH₃), 2.43 (s, 3H, -CH₃), 2.39 (s, 3H, -CH₃), 2.37 (s, 3H, -CH₃). Anal. Calcd. for C₁₆H₁₇N₃S₂: C, 60.92; H, 5.43; N, 13.32; S, 20.33. Found: C, 60.71; H, 5.63; N, 13.19; S, 20.19.

N,2,5,6-tetramethyl-N-(4-(methylthio)phenyl)thieno[2,3-d]pyrimidin-4-amine (226)

Compound 228 (220 mg, 0.734 mmol) was dissolved in DMF (10 ml) followed by portion wise addition of sodium hydride (60% dispersed in mineral oil) (29.4 mg, 0.734 mmol). The reaction was stirred at room temperature for 10 mins and then iodomethane (0.55 mL, 0.881 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane. The compound was tritutated in diethyl ether to further purify and afford 226 as a yellow solid (83 mg, 36% yield). TLC Rf = 0.55 (Hexane: EtOAc, 3:1); mp, 190-193 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.17 (d, J = 8.3 Hz, 2H, Ar), 6.86 (d, J = 8.3 Hz, 2H, Ar), 3.46 (s, 3H, -N⁴CH₃), 2.59 (s, 3H, S-CH₃), 2.43 (s, 3H, 2-CH₃), 2.29 (s, 3H, 6-CH₃), 1.62 (s, 3H, 5-CH₃). Anal. Calcd. for C₁₇H₁₉N₃S₂ 0.22 (C₂H₅)₂O: C, 62.11; H, 6.18; N, 12.14; S, 18.53. Found: C, 62.16; H, 5.80; N, 12.18; S, 18.35.
7-methoxy-4-(2,5,6-trimethylthieno[2,3-d]pyrimidin-4-yl)-3,4-dihydro-2H-benzo[b][1,4]oxazine (229)

To a solution of 318 (200 mg, 0.940 mmol) in isopropanol (10 mL), 1 drop of HCl was added, followed by addition of 7-methoxy-3,4-dihydro-2H-benzo[b][1,4]oxazine (170.87 mg, 1.03 mmol) and refluxed for 8 hours. The reaction mixture was cooled to room temperature, silica gel was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using hexane and ethyl acetate (10:1) to give an off-white solid as 229 (186 mg, 58% yield). TLC $R_f = 0.20$ (Hexane: EtOAC, 3:1); mp, 210-211.8 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.98 (d, $J = 3.1$ Hz, 1H, Ar), 6.79 (dd, $J = 8.9$ Hz, $J = 3.1$ Hz, 1H, Ar), 6.32 (d, $J = 8.9$ Hz, 1H, Ar), 3.99 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 3.87 (t, $J = 6.5$ Hz, 2H, -CH$_2$-), 3.82 (s, 3H, -OCH$_3$), 2.57 (s, 3H, 2-CH$_3$), 2.31 (s, 3H, 6-CH$_3$), 1.65 (s, 3H, 5-CH$_3$). Anal. Calcd. for C$_{18}$H$_{19}$N$_3$O$_2$S: C, 63.32; H, 5.61; N, 12.30; S, 9.39. Found: C, 63.30; H, 5.71; N, 12.22; S, 9.22.

2-Cyano-3-phenylpropenal (321)

Benzaldehyde 319 (5.0 g, 0.047 mmol) and 3,3-dimethoxypropanenitrile (7.23 g, 0.062 mmol) were mixed together and added to a solution of NaOMe (3.39 g, 0.062 mmol) in MeOH (15 mL) during 15 min. The mixture was stirred at room temperature overnight. Most of the MeOH was removed in vacuo, and the residue was partitioned between EtOAc (50 mL) and water (50 mL). The organic layer was separated, washed with brine (40 mL), and dried (MgSO$_4$) and the solvent evaporated in vacuo. The residual oil was distilled to give 320 (8.43 g, 66% yield) as a colorless oil. The residual oil (8.43 g, 0.041 mmol) without further purification and characterization was treated cautiously with 6 N HCl (5.7 mL), and the mixture was stirred at room temperature for 30 mins. The solid was filtered off, washed well with water, and dried in vacuo to give 321 as an off-white powder (3.65 g, 56% yield). TLC $R_f = 0.20$ (Hexane: EtOAC, 3:1); mp, 98 °C (lit. $^{240}$ 96-97
\( ^\circ \text{C} \); \( ^1\text{H} \text{NMR} (400 \text{ MHz, DMSO-}d_6) \delta 9.62 \text{ (s, 1H, -CHO), 8.53 (s, 1H, -CH), 8.05 (d, } J = 9.4 \text{ Hz, 2H, Ar), 7.78 – 7.61 (m, 3H, Ar). Anal. Calcd. for C_{10}H_7NO: C, 76.42; H, 4.49; N, 8.91. Found C, 76.03; H, 4.73; N, 8.78. \\

**Methyl 3-amino-4-benzyl-1\text{H}-pyrrole-2-carboxylate (323)**

Synthesis of 323 was initiated by reacting 321 (3.50 g, 22.27 mmol) in methanol with 10% Pd/C under 50 psi H\(_2\) in a hydrogenation bottle for 3 hours at room temperature. The catalyst was removed by filtration to obtain crude 322. Crude 322 (2.8 g, 17.6 mmol) was added with diethyl aminomalonate (5.67 g, 0.03 mmol) and sodium acetate (2.19 g, 0.03 mmol) and water (5 mL) was added, and the mixture was stirred at room temperature for 16 hours. Most of the solvent was removed in vacuo, and the residue was partitioned between EtOAc (25 mL) and water (20 mL). The organic layer was separated and dried over sodium sulphate and evaporated in vacuo. The residual yellow oil was dissolved in methanol containing sodium methoxide (0.48 g, 0.01 mmol), stirred at room temperature for 12 hours and then heated to reflux for 3 hours. Most of the solvent was evaporated in vacuo, and the residue was treated with water (20 mL) and was flash chromatographed with 1% (v/v) CH\(_3\)OH in CHCl\(_3\) to give 323 as a light-yellow solid (1.38 g, 34% over 3 steps). TLC \( R_f = 0.65 \) (CHCl\(_3\): CH\(_3\)OH, 10:1); mp, 121-123.2 \( ^\circ \text{C} \) (lit.\(^{240} 120-122 \text{ °C} \)); \( ^1\text{H} \text{NMR} (400 \text{ MHz, DMSO-}d_6) \delta 10.47 \text{ (br, 1H, exch., NH), 7.19 - 7.27 (m, 4 H, Ar), 7.11 - 7.16 (m, 1 H, Ar), 6.46 (d, } J = 3.51 \text{ Hz, 1 H, 6-H), 4.84 (br, 2H, exch., NH}_2\text{), 3.68 (s, 3 H, -CH}_3\text{), 3.62 (s, 2H, -CH}_2\text{). This compound was used for the next reaction without further characterization.
7-benzyl-N-(4-methoxyphenyl)-N-methyl-5H-pyrrolo[3,2-d]pyrimidin-4-amine (232)

Treatment 323 (1.0 g, 4.34 mmol) with 3.46 mL (86.86 mmol) formamide was carried out in microwave vessel at 170 °C for 12 hours. The reaction was cooled to room temperature and to the mixture, 50 mL water was added, and the precipitate was collected and dried under high vacuum to afford 324 as white solid. (636 mg, 65 % yield). The product 324 was not subjected to separation efforts and taken to the next step without any characterization. Chlorination of 324 (600 mg, 2.66 mmol) was performed using POCl₃ (0.25 mL, 2.66 mmol) and pyridine (0.2 mL, 2.66 mmol) in toluene and refluxed for 4 hours. The solvent was evaporated and neutralized with ammonia in water solution to generated pale-yellow precipitate. The precipitate was collected by filtration. To the precipitate, was added methanol and 2.0 g of silica gel. The solvent was reduced under pressure and silica plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 325 as a white solid (440 mg, 68% yield). It was used in the next reaction, without further separation or characterization efforts. Compound 325 (150 mg, 0.615 mmol) was dissolved in isopropanol (15 mL) followed by addition of 1 drop of HCl and 4-methoxy-N-methylaniline (93 mg, 0.677 mmol) refluxed for 3 hours. The solvent was evaporated in vacuo and silica gel and methanol were added after that the solvent was evaporated to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with 1% (v/v) CH₃OH/CHCl₃. Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford 232 (152.5 mg, 72% yield). TLC Rf 0.39 (CHCl₃:CH₃OH:NH₄OH; 20:1:0.5); mp, 196-198.2 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.57 (br, 1H, exch, NH), 8.34 (s, 1H, Ar), 7.4 (d, 2H, J = 8.72, Ar), 7.17 – 7.26 (m, 6H, Ar and 6-CH), 7.08 (d, 2H, J = 8.72 Hz, Ar), 3.95 (s, 2H, -CH₂-), 3.80 (s, 3H, -OCH₃), 3.45 (s, 3H, -N(CH₃)₃). Anal. Calcd. for C₂₁H₂₀N₄O 0.09 (CH₃)₂CHOH 0.03 HCl: C, 72.77; H, 5.97; N, 15.94. Found C, 72.76; H, 6.03; N, 15.98.
Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (330)

4-Cyclohexanone 331 derivatives (5.27 mL, 50.95 mmol) and morpholine (4.39 mL, 50.95 mmol) were added to the mixture of ethyl cyanoacetate (5.42 mL, 50.95 mmol) and sulfur (13.07 g, 50.95 mmol) in ethanol (25 mL). Then the mixture was stirred at room temperature for 1 h then stirred at 60 °C for 12 h. The reaction mixture was cooled to room temperature and the solvent was removed in vacuo. The crude product was purified by flash column chromatography on a silica column using hexane/ethyl acetate (10:1) as the eluent to get compound 330 (7.81 g, 68% yield) as a light-yellow solid. TLC $R_f = 0.67$ (Hexane: EtOAc, 3:1); mp, 194-195.7 °C (lit.237 192-193 °C); $^1$H NMR (400 MHz, DMSO-$d_6$) δ 6.66 (s, 2H, br, exch., NH$_2$), 4.36 (q, $J = 7.0$ Hz, 2H, -CH$_2$CH$_3$), 2.60 (m, 2H, -CH$_2$), 2.41 (m, 2H, -CH$_2$), 1.68-1.65 (m, 4H, -CH$_2$), 1.42 (t, $J = 7.0$ Hz, 3H, -CH$_2$CH$_3$). The $^1$H-NMR matches the $^1$H-NMR of the reported compounds in the literature.237 This compound was used for the next reaction without further characterization.

2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (329)

Compound 330 (2.5 g, 11.10 mmol) was dissolved in 20 mL of acetonitrile and hydrogen chloride gas was bubbled through for 30 mins. The mixture was stirred at room temperature overnight. The residue was dried and dissolved in 10 mL distilled water and then treated with ammonia in water solution to generated white precipitate. The precipitate was collected by filtration to afford 1.27 g (52% yield) of 329 as a white solid. TLC $R_f = 0.11$ (Hexane: EtOAc, 3:1); mp $>250$ °C (lit. $>250$ °C)404 $^1$H NMR (400 MHz, DMSO-d6): δ 12.10 (s, 1H, exch., -NH), 3.02-2.95 (m, 2H, -CH$_2$-), 2.87-2.84 (m, 2H, -CH$_2$-), 2.66 (s, 3H, 2-CH$_3$), 1.84 (t, $J=$
3.1 Hz, 4H, -CH₂-). This compound was used for the next reaction without further characterization.

**4-chloro-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (328)**

Chlorination of 329 (1.0 g, 4.54 mmol) was carried out using POCl₃ (0.4 mL, 4.54 mmol), pyridine (0.37 mL, 4.54 mmol) in toluene in reflux condition for 6 hours. The solvent was evaporated and neutralized with ammonia in water solution to generated light-yellow precipitate. The precipitate was collected by filtration to afford 328 as light-yellow solid (704.42 mg, 65% yield). TLC $R_f = 0.83$ (Hexane: EtOAc, 3:1); mp, 228-230 °C; $^1$H NMR (400 Hz) (Me₂SO-d₆) δ 2.79-2.85 (m, 2H, -CH₂-), 2.84-2.80 (m, 2H, -CH₂-), 2.58 (s, 3H, -2-CH₃), 1.81-1.77 (m, 4H, -CH₂-). This compound was used for the next reaction without further characterization.

**General procedure for synthesis of 234, 236, 238, 239 and 240**

Compound 328 was dissolved in isopropanol followed by adding 1-2 drops of HCl and appropriate anilines. The reaction mixture was stirred for 4-8 hours at reflux. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 234, 236, 238, 239 and 240 (yields: 55-71%).
N-(4-methoxyphenyl)-N,2-dimethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-
derived-pyrimidin-4-amine (234)

Compound 328 (250 mg, 1.05 mmol) was dissolved in isopropanol (10 mL) followed by
addition of 1-2 drops of HCl and reacted with p-anisidine (0.2 mL, 1.36 mmol) for 4 hours at
reflux conditions. The reaction mixture was cooled, and silica gel was added to the solvent
mixture and plug was prepared. A flash column chromatographic separation was performed
using ethyl acetate-hexane as eluent to afford intermediate 334 as a white solid (151 mg, 40%
yield); TLC \( R_f = 0.22 \) (Hexane: EtOAc, 1:1). Crude 334 (220 mg, 0.676 mmol) without
further characterization was dissolved in DMF (10 ml) followed by portion wise addition of
sodium hydride (60% in dispersion, 27.04 mg, 0.676 mmol). Iodomethane (0.04 mL, 0.676
mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was
stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug
was prepared. A flash column chromatographic separation was performed using ethyl acetate
hexane as eluent to afford 234 as a white solid (156 mg, 68% yield); TLC \( R_f = 0.41 \) (Hexane:
EtOAc, 3:1); mp, 198-199.6 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 6.92 (d, \( J = 9.1 \) Hz, 2H,
Ar), 6.86 (d, \( J = 9.1 \) Hz, 2H, Ar), 3.72 (s, 3H, -OCH\(_3\)), 3.42 (s, 3H, -N\(^4\)CH\(_3\)), 2.70 (m, 2H,-
CH\(_2\)), 2.56 (s, 3H, 2-CH\(_3\)), 1.72 (m, 2H, -CH\(_2\)), 1.60 – 1.52 (m, 2H, -CH\(_2\)), 1.46 – 1.34 (m,
2H, -CH\(_2\)). Anal. Calcd. for C\(_{19}\)H\(_{21}\)N\(_3\)OS: C, 67.22; H, 6.24; N, 12.37; S, 9.45. Found: C,
67.38; H, 6.23; N, 12.31; S, 9.44.
\(N,2\text{-dimethyl-}N\text{-}(4\text{-}(methylthio)phenyl)-5,6,7,8\text{-tetrahydrobenzo[4,5]thieno[2,3-}d\text{]}\text{pyrimidin-4-amine} (236)\)

Compound 328 (300 mg, 1.26 mmol) was dissolved in isopropanol followed by addition of 1-2 drops of HCl and reacted with 4-(methylthio)aniline (0.2 mL, 1.63 mmol) for 6 hours at reflux condition. The reaction mixture was cooled down and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford intermediate 335 as a pale-yellow solid (186 mg, 41% yield); TLC \(R_f = 0.20\) (Hexane: EtOAc, 1:1). Crude 335 (150 mg, 0.439 mmol) without further characterization was dissolved in DMF (10 mL) followed by portion wise addition of sodium hydride (17.6 mg, 0.439 mmol). Iodomethane (0.03 mL, 0.439 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 236 as a yellow solid (109 mg, 70% yield); TLC \(R_f = 0.45\) (Hexane: EtOAc, 3:1); mp, 189-191 °C; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta 7.16 \text{ (d, } J = 8.8 \text{ Hz, 2H, Ar), 6.85 \text{ (d, } J = 8.8 \text{ Hz, 2H, Ar), 3.41 \text{ (s, 3H, } -N^4\text{CH}_3), 2.69 \text{ (m, 2H, -CH}_2\text{), 2.55 \text{ (s, 3H, -CH}_3\text{), 2.41 \text{ (s, 3H, -SCH}_3\text{), 1.83 \text{ (m, 2H, -CH}_2\text{), 1.62 – 1.50 \text{ (m, 2H, -CH}_2\text{), 1.46 – 1.37 \text{ (m, 2H, -CH}_2\text{). Anal. Calcd. for C}_{19}H_{21}N_3S_2: C, 64.19; H, 5.95; N, 11.82; S, 18.04. Found: C, 64.44; H, 5.99; N, 11.70; S, 17.78.} \)
4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (238)

Compound 328 (250 mg, 1.05 mmol) was dissolved in isopropanol (15 mL) followed by adding 1-2 drops of HCl and 6-methoxy-1,2,3,4-tetrahydroquinoline (192 mg, 1.20 mmol). The reaction mixture was stirred for 8 hours at reflux condition. The reaction mixture was cooled, and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using hexane and EtOAc as eluent to afford 238 as a yellow solid (209 mg, 55% yield); TLC Rf = 0.53 (Hexane: EtOAc, 3:1); mp, 199-201 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) δ 6.78 (d, J = 3.1 Hz, 1H, Ar), 6.54 (dd, J = 8.8 Hz, J = 3.1 Hz, 1H, Ar), 6.34 (d, J = 8.8 Hz, 1H, Ar), 3.80-3.71 (m, 2H, -CH$_2$), 3.69 (s, 3H, -OCH$_3$), 3.48-3.42 (m, 2H, -CH$_2$), 2.82-2.70 (m, 4H, -CH$_2$), 2.54 (s, 3H, 2-CHOH): C, 68.46; H, 6.77; N, 10.79; S, 8.24. Found: C, 68.78; H, 6.39; N, 10.52; S, 7.91.

$N$-(5-methoxynaphthalen-2-yl)-$N$,2-dimethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (239)

Compound 328 (300 mg, 1.26 mmol) was dissolved in isopropanol followed by addition of 1-2 drops of HCl and reacted with 6-aminonaphthalen-1-ol (220 mg, 1.38 mmol) for 6 hours at reflux condition. The reaction mixture was cooled down and silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford intermediate 240 as a pale-yellow solid (322.5 mg, 71% yield); TLC Rf = 0.21 (Hexane: EtOAc, 3:1); mp, 214 °C. Without further characterization compound 240 (250 mg, 0.691 mmol) was dissolved in DMF (12 mL) followed by portion wise addition of
sodium hydride (60% in dispersion, 27.6 mg, 0.691 mmol). Iodomethane (0.04 mL, 0.691 mmol) dissolved in 5 mL of DMF was added drop wise to the suspension. The reaction was stirred for 2 hours at room temperature. Silica gel was added to the solvent mixture and plug was prepared. A flash column chromatographic separation was performed using ethyl acetate hexane as eluent to afford 239 as a yellow solid (153.5 mg, 57% yield); TLC $R_f = 0.46$ (Hexane: EtOAc, 3:1); mp, 205-208 °C; $^1$H NMR (400 MHz, DMSO- $d_6$) $\delta$ 7.99 (d, $J = 9.2$ Hz, 1H, Ar), 7.32 (d, $J = 7.8$ Hz, 1H, Ar), 7.29 – 7.24 (m, 2H, Ar), 7.09 (dd, $J = 9.2$ Hz, $J = 2.4$ Hz, 1H, Ar), 6.82 (dd, $J = 7.8$ Hz, $J = 3.0$ Hz, 1H, Ar), 3.92 (s, 3H, -OCH$_3$), 3.53 (s, 3H, -N$^4$CH$_3$), 2.69 (t, $J = 6.08$ Hz, 2H, -CH$_2$), 2.61 (s, 3H, 2-CH$_3$), 1.87 (t, $J = 6.08$ Hz, 2H, -CH$_2$), 1.51 – 1.45 (m, 2H, -CH$_2$), 1.40 – 1.32 (m, 2H, -CH$_2$). Anal. Calcd. for C$_{23}$H$_{23}$N$_3$OS: C, 70.92; H, 5.95; N, 10.78; S, 8.23. Found: C, 70.75; H, 6.11; N, 10.53; S, 7.95.

2-amino-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (336)

Methyl sulfone (15 g), compound 330 (5.0 g, 22.19 mmol) and chloroformamidine hydrochloride (5.10 g, 44.38 mmol) were taken in a round bottom flask. The reaction mixture was stirred at 140 °C for 4 hours. The reaction was quenched with 100 mL water, cooled in an ice bath and was basified to pH 8.0 using an aqueous NH$_4$OH solution. The precipitate was collected by filtration, dried (using Na$_2$SO$_4$) and afforded 3.24 g (66%) of 336 as a brown solid. TLC $R_f = 0.60$ (CHCl$_3$: MeOH, 5:1); The product 336 was not subjected to separation efforts and taken to the next step without any characterization.
N-(4-chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-2-yl)pivalamide (338)

To a 250 mL round bottom flask were added 336 (3.0 g, 13.56 mmol) and Piv₂O (25 mL, 135.58 mmol) and the reaction mixture was vigorously stirred at reflux condition. After 20 min, the reactant was completely dissolved, and, after 1 h, the product precipitated. After 2 h, TLC indicated complete disappearance of 336. The reaction mixture was cooled to room temperature, and the solid was collected by filtration, washed with hexane and dried to give 2.90 g (70% yield) of 337 as a brown solid. TLC \( R_f = 0.38 \) (Hexane: EtOAc, 3:1); mp 256-258 °C; This compound was used for the next reaction without further characterization. Crude 337 (2.70 g, 8.84 mmol) was chlorinated with phosphorus oxychloride (0.8 mL, 8.84 mmol) and pyridine (0.7 mL, 8.84 mmol) in toluene (15 mL). The reaction was refluxed for 4 hours. The POCl₃ was evaporated, and the mixture was cooled in ice bath. The mixture was neutralized using an aqueous NH₄OH solution to yield a precipitate. The precipitate was collected by filtration, washed with water, dried and then dissolved in MeOH. To the solution was added silica gel (1 g), and the solvent was removed under reduced pressure to provide a silica gel plug. Column chromatography was performed with hexane and ethyl acetate (10:1) to generate 338 (1.49 g, 52%) as a brown solid. TLC \( R_f = 0.68 \) (Hexane: EtOAc, 3:1); mp 234-235.6 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \( \delta \) 2.85-2.80 (m, 2H, -CH₂), 2.71-2.65 (m, 2H, -CH₂), 1.80 – 1.71 (m, 4H, -CH₂), 1.24 (s, 9H, -Piv). This compound was used for the next reaction without further characterization.
**N4-(4-methoxyphenyl)-N4-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine-2,4-diamine (244)**

To a solution of 338 (250 mg, 0.771 mmol) in isopropanol (20 mL), 1 drop of HCl was added, followed by addition of 4-methoxy-N-methylaniline (116.5 mg, 0.849 mmol) under reflux condition for 6 hours. After cooling to room temperature, 1 N NaOH solution (2 mL) was added and heated to reflux for 2 h. The reaction mixture was cooled to rt, silica gel was added, and the solvent was removed under reduced pressure. Purification was performed by flash column chromatography using 1% MeOH in CHCl₃ as the eluent, and the fractions containing the product (TLC) were pooled, evaporated to give a solid which was then washed with CHCl₃ to afford 147 mg (56% in two steps) of 244 as white solid. TLC R_f = 0.40 (CHCl₃: MeOH, 20:1); mp 167 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 6.93 (d, J = 9.0 Hz, 2H, Ar), 6.82 (d, J = 9.0 Hz, 2H, Ar), 5.19 (s, 2H, exch., 2-NH₂), 3.81 (s, 3H, -OCH₃), 3.46 (s, 3H, -N₄CH₃), 2.67-2.63 (m, 2H, -CH₂), 1.66-1.60 (m, 4H, -CH₂CH₂), 1.49-1.45 (m, 2H, -CH₂). Anal. Calcd. for C₁₈H₂₀N₄OS 0.29 H₂O: C, 62.54; H, 6.00; N, 16.20; S, 9.27. Found: C, 62.58; H, 6.03; N, 16.10; S, 9.21.

**N4-methyl-N4-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine-2,4-diamine (245)**

To a solution of 338 (220 mg, 0.679 mmol) in isopropanol (20 mL), 1 drop of HCl was added, followed by addition of N-methyl-4-(methylthio)aniline (114.5 mg, 0.747 mmol) under reflux condition for 6 hours. After cooling to room temperature, 1 N NaOH solution (2 mL) was added and heated to reflux for 2 h. The reaction mixture was cooled to room temperature, silica gel was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using 1% MeOH in CHCl₃ as the eluent, and the fractions containing the
product (TLC) were pooled, evaporated to give a solid which was then washed with CHCl₃ to afford 116 mg (48% in two steps) of 245 as pale yellow solid. TLC Rᶠ = 0.35 (CHCl₃: MeOH, 20:1); mp 172-173.5 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.12 (d, J = 9.0 Hz, 2H, Ar), 6.97 (d, J = 9.0 Hz, 2H, Ar), 5.53 (s, br, 2H, exch., 2-NH₂), 3.51 (s, 3H, -N⁴CH₃), 2.39 (s, 3H, -SCH₃), 2.68-2.64 (m, 2H, -CH₂-), 1.67-1.59 (m, 4H, -CH₂), 1.48 – 1.44 (m, 2H, -CH₂). Anal. Calcd. for C₁₈H₂₀N₄S₂ 0.18 CH₃OH: C, 60.26; H, 5.77; N, 15.46; S, 17.69. Found: C, 60.24; H, 5.82; N, 15.47; S, 17.72.

4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-d] pyrimidin-2-amine (246)

To a solution of 338 (300 mg, 0.926 mmol) in isopropanol (20 mL), 1-2 drops of HCl was added, followed by addition of 6-methoxy-1,2,3,4-tetrahydroquinoline (166.3 mg, 1.02 mmol) under reflux condition for 8 hours. After cooling to room temperature, 1 N NaOH solution (2 mL) was added and heated to reflux for 2 h. The reaction mixture was cooled to room temperature, silica gel (500 mg) was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using 1% MeOH in CHCl₃ as the eluent, and the fractions containing the product (TLC) were pooled, evaporated to give a solid which was then washed with CHCl₃ to afford 142.6 mg (42% in two steps) of 246 as an off-white solid. TLC Rᶠ = 0.32 (CHCl₃: MeOH, 20:1); mp 190-191.8 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 6.79 (d, J = 3.0 Hz, 1H, Ar), 6.56 (dd, J = 8.9 Hz, J = 3.0 Hz, 1H, Ar), 6.39 (d, J = 8.9 Hz, 1H, Ar), 5.18 (s, br, 2H, exch., 2-NH₂), 3.95-3.88 (m, 2H, -CH₂), 3.82 (s, 3H, -OCH₃), 2.75 (m, 2H, -CH₂), 2.68-2.64 (m, 2H, -CH₂), 1.94-1.88 (m, 2H, -CH₂), 1.68-1.61 (m, 4H, -CH₂), 1.49-1.45 (m, 2H, -CH₂). MS (ESI) m/z calculated for C₂₀H₂₂N₄OS [M+H]⁺, 367.48. Found: 366.8. HPLC analysis: retention time, 13.63
min; peak area, 95.51%; eluent A, H₂O; eluent B, ACN; gradient elution (100% H₂O to 10% H₂O) over 60 min with flow rate of 0.5 mL/min and detection at 245 nm; column temperature, rt.

4-chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (340)

Treatment 330 (5.0 g, 22.19 mmol) with formamide (4.42 mL, 110.96 mmol) was carried out in microwave vessel at 180 °C for 12 hours. The reaction was cooled to room temperature and to the mixture, 50 mL water was added, and the precipitate was collected and dried under high vacuum to afford 339 as white solid in 72% yield (3.30 g). The product 339 was not subjected to separation efforts and taken to the next step without any characterization. Chlorination of 339 (3.0 g, 14.54 mmol) was performed using POCl₃ (1.4 mL, 14.54 mmol) and pyridine (1.17 mL, 14.54 mmol) and refluxed for 8 hours. The solvent was evaporated and neutralized with ammonia in water solution to generated pale-yellow precipitate. The precipitate was collected by filtration. To the precipitate, was added methanol and silica gel. The solvent was reduced under pressure and silica plug was prepared. A flash column chromatographic separation was performed using ethyl acetate-hexane as eluent to afford 1.96 g of 340 (60% yield) as pale-yellow solid. TLC Rf = 0.77 (Hexane: EtOAc, 3:1); mp, 210-112 °C; ¹H NMR (400 Hz) (Me₂SO-d₆) δ 8.51 (s, 1H, Ar), 2.78-2.84 (m, 2H, -CH₂), 2.86-2.82 (m, 2H, -CH₂), 1.84-1.78 (m, 4H, -CH₂). Anal. Calcd. for C₁₀H₉ClN₂S: C, 53.45; H, 4.04; Cl, 15.78, N, 12.47; S, 14.27. Found: C, 53.58; H, 4.07; Cl, 15.57, N, 12.37; S, 14.12.
**N-(4-methoxyphenyl)-N-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (247)**

To a solution of 340 (200 mg, 0.890 mmol) in isopropanol (15 mL), 1-2 drops of HCl was added, followed by addition of 4-methoxy-N-methylaniline (134.3 mg, 0.979 mmol) under reflux condition for 6 hours. The reaction mixture was cooled to room temperature, silica gel was added, and the solvent was removed under reduced pressure. Purification was performed by flash column chromatography using hexane and ethyl acetate (10:1) to give 247 as white solid (197 mg, 68% yield). TLC $R_f = 0.48$ (Hexane: EtOAc, 3:1); mp 186-188 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.58 (s, 1H, Ar), 6.93 (d, $J = 9.1$ Hz, 2H, Ar), 6.86 (d, $J = 9.1$ Hz, 2H, Ar), 3.71 (s, 3H, -OCH$_3$), 3.42 (s, 3H, -N$_4$CH$_3$), 2.72 (m, 2H, -CH$_2$), 1.74 (m, 2H, -CH$_2$), 1.61 – 1.50 (m, 2H, -CH$_2$), 1.46 – 1.35 (m, 2H, -CH$_2$). Anal. Calcd. for C$_{18}$H$_{19}$N$_3$OS: C, 66.43; H, 5.88; N, 12.91; S, 9.85. Found: C, 66.72; H, 5.79; N, 12.88; S, 9.66.

**N-methyl-N-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (248)**

To a solution of 340 (150 mg, 0.667 mmol) in isopropanol (12 mL), 1-2 drops of HCl was added, followed by addition of N-methyl-4-(methylthio)aniline (112.5 mg, 0.734 mmol) under reflux condition for 6 hours. The reaction mixture was cooled to room temperature, silica gel was added, and the solvent was removed under reduced pressure. Purification was performed by column chromatography using hexane and ethyl acetate (10:1) to give 248 as light-yellow solid (160 mg, 70% yield). TLC $R_f = 0.40$ (Hexane: EtOAc, 3:1); mp, 192-193.6 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.55 (s, 1H, Ar), 6.91 (d, $J = 9.1$ Hz, 2H, Ar), 6.82 (d, $J = 9.1$ Hz, 2H, Ar), 3.51 (s, 3H, -N$_4$CH$_3$), 2.40 (s, 3H, -SCH$_3$), 2.74 (m, 2H, -CH$_2$), 1.76 (m, 2H, -CH$_2$), 1.60 – 1.54 (m, 2H, -CH$_2$),
1.46 – 1.39 (m, 2H, -CH₂). Anal. Calcd. for C₁₈H₁₉N₃S₂ 0.32 (CH₃)₂CHOH 0.05 HCl: C, 62.84; H, 6.02; N, 11.59; S, 17.68. Found: C, 62.68; H, 5.79; N, 11.72; S, 17.49.

**Methyl 1-methyl-4-nitro-1H-pyrazole-5-carboxylate (342a)**

In a solution of DMF (25 mL) commercially available methyl 4-nitro-1H-pyrazole-5-carboxylate 341 (10 g, 58.44 mmol) was reacted with K₂CO₃ and methyl iodide (5.5 mL, 87.66 mmol) at room temperature for 12 hours. A flash column chromatography using ethyl acetate-hexane afforded two regioisomers: methyl 1-methyl-4-nitro-1H-pyrazole-5-carboxylate (342a) in 35% (3.8 g) as colorless semi solid and methyl 1-methyl-4-nitro-1H-pyrazole-3-carboxylate (342b) in 55% (6.0 g) yield as white solid. The structures of the isomers (342a and 342b) were determined by NOESY analysis: a NOESY correlation was observed between H-5 and N-1 alkyl protons in 342b which was absent in 342a. TLC $R_f$ = 0.40 (Hexane: EtOAc, 3:1); $^1$H NMR (400 MHz, DMSO-$_d_6$)  δ 8.36 (s, 1H, Ar), 4.02 (s, 3H, N1-CH₃), 3.98 (s, 3H, -OCH₃). The $^1$H-NMR matches the $^1$H-NMR of the reported compounds in the literature. This compound was used for the next reaction without further characterization.

**Methyl 1-methyl-4-nitro-1H-pyrazole-3-carboxylate (342b)**

Methyl 1-methyl-4-nitro-1H-pyrazole-3-carboxylate 342b was isolated as white solid in 55% yield as white solid. TLC $R_f$ = 0.50 (Hexane: EtOAc, 3:1); mp, 128.1-129.2 °C; $^1$H NMR (400 MHz, DMSO-$_d_6$)  δ 8.95 (s, 1H, Ar), 3.95 (s, 3H, N1-CH₃), 3.89 (s, 3H, -OCH₃). The $^1$H-NMR matches the $^1$H-NMR of the reported compounds in the literature. Anal. Calcd. for C₆H₇N₃O₄: C, 38.92; H, 3.81; N, 22.69. Found: C, 39.04; H, 3.79; N, 22.51.
5,7-dichloro-1-methyl-1H-pyrazolo[4,3-d]pyrimidine (344)

Reduction of 342a (2.7 g, 14.58 mmol) using 10% Pd/C in EtOH produced 343 (1.63 g, 72% yield) which was finely mixed with urea (2.52 g, 42.02 mmol) without solvent in a microwave vial and heated at 180 °C for 2 hours. During the reaction course, complete fusion followed by re-solidification was observed. The crude product was purified by crystallization from 10% NaOH and was carried out to next step without any column separation and further characterization. Chlorination of crude (1.47 g, 8.85 mmol) was carried out using POCl₃ (0.8 mL, 8.85 mmol) in toluene (20 mL) and pyridine (0.7 mL, 8.85 mmol) and refluxed for 4 hours. The solvent was evaporated and cooled in an ice bath followed by neutralization with ammonia in water solution. White precipitate was formed. The resulting precipitate was filtered and purified by column chromatography to afford 344 as pale-yellow solid. (880 mg, 42% in two steps). TLC Rf = 0.62 (Hexane: EtOAc, 3:1); mp, 168-169 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.46 (s, 1H, Ar), 4.33 (s, 3H, N₁-CH₃). The ¹H-NMR matches the ¹H-NMR of the reported compounds in the literature. This compound was used for the next reaction without further characterization.

5-chloro-N-(4-methoxyphenyl)-N₁,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (251)

To a solution of 344 (0.2 g, 0.985 mmol) in acetonitrile (15 mL) was added 4-methoxy-N-methylaniline (162.16 mg, 1.18 mmol) and the resulting mixture was refluxed for 12 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc (10:1) to give white solid 251 (113 mg, 38%). TLC Rf = 0.20 (Hexane: EtOAc, 3:1); mp, 123.5-124.6 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.05 (s, 1H, Ar), 7.25 (d, J = 8.9 Hz, 2H, Ar), 7.00 (d, J = 8.9 Hz, 2H, Ar), 3.79 (s, 3H, OCH₃), 3.49 (s, 3H, N₇-CH₃), 3.04 (s, 3H, N₁-CH₃). Anal. Calcd. for C₁₄H₁₄ClN₅O 0.05
CH₃(CH₂)₄CH₃: C, 55.73; H, 4.79; Cl, 11.52; N, 22.76. Found: C, 55.84; H, 4.77; Cl, 11.28; N, 23.00.

5-chloro-N-(4-methoxyphenyl)-1-methyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (252)

To a solution of 344 (0.2 g, 0.985 mmol) in acetonitrile (15 mL) was added 4-methoxyaniline (145.58 mg, 1.18 mmol) and the resulting mixture refluxed for 8 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc 4:1 to give a white solid 252 (128 mg, 45%). TLC Rf = 0.25 (Hexane: EtOAc, 1:1); mp, 118-120.2 °C; ¹H NMR (500 MHz, DMSO-d₆) δ 9.08 (s, 1H, exch., N⁷-H), 8.02 (s, 1H, Ar), 7.50 (d, J = 9.3 Hz, 2H, Ar), 7.02 (d, 2H J = 9.3 Hz, Ar), 4.35 (s, 3H, N₁-CH₃), 3.79 (s, 3H, -OCH₃). Anal. Calcd. for C₁₃H₁₂ClN₅O: C, 53.89; H, 4.18; Cl, 12.24; N, 24.17. Found: C, 53.80; H, 4.16; Cl, 12.31; N, 24.23.

1-(5-chloro-1-methyl-1H-pyrazolo[4,3-d]pyrimidin-7-yl)-6-methoxy-1,2,3,4-tetrahydroquinoline (253)

To a solution of 344 (0.25 g, 1.23 mmol) in acetonitrile (15 mL) was added 6-methoxy-1,2,3,4-tetrahydroquinoline (241.18 mg, 1.48 mmol) and the resulting mixture was refluxed for 12 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc to give an off-white solid 253 (162 mg, 40%). TLC Rf = 0.28 (Hexane: EtOAc, 3:1); mp, 162.4-164.3 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.12 (s, 1H, Ar), 6.93 (d, J = 3.0 Hz, 1H, Ar), 6.74 (dd, J = 8.2 Hz, J = 3.0 Hz 1H, Ar), 6.70 (d, J = 8.2 Hz, 1H, Ar), 3.94-3.83 (m, 2H, -CH₂), 3.75 (s, 3H, -OCH₃), 3.21 (s, 3H, N₁-CH₃), 2.85 – 2.78 (m, 2H, -CH₂), 2.08 – 1.99 (m, 2H, -CH₂). Anal. Calcd. for C₁₆H₁₂ClN₅O
5-chloro-N-(4-ethoxyphenyl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (257)

To a solution of 344 (0.2 g, 0.985 mmol) in acetonitrile (15 mL) was added 4-ethoxy-N-methylaniline (178.75 mg, 1.18 mmol) and the resulting mixture was refluxed for 10 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc to give off white solid 257 (131.5 mg, 42%). TLC Rf = 0.55 (Hexane: EtOAc, 3:1); mp, 144-146 °C; 1H NMR (500 MHz, DMSO-d$_6$) δ 8.04 (s, 1H, Ar), 7.21 (d, J = 9.0 Hz, 2H, Ar), 6.98 (d, J = 9.0 Hz, 2H, Ar), 4.04 (q, J = 6.9 Hz, 2H, -OCH$_2$CH$_3$), 3.48 (s, 3H, N$^7$-CH$_3$), 3.03 (s, 3H, N1-CH$_3$), 1.32 (t, J = 6.9 Hz, 3H, -OCH$_2$CH$_3$). Anal. Calcd. for C$_{15}$H$_{16}$ClN$_5$O: C, 56.69; H, 5.08; Cl, 11.16; N, 22.04. Found: C, 56.76; H, 5.18; Cl, 11.07; N, 21.91.

5-chloro-N-(4-isopropoxyphenyl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (258)

TLC Rf = 0.42 (Hexane: EtOAc, 3:1); mp, 162-163.8 °C; 1H NMR (500 MHz, DMSO-d$_6$) δ 8.05 (s, 1H, Ar), 7.21 (d, J = 9.0 Hz, 2H, Ar), 6.98 (d, J = 9.0 Hz, 2H, Ar), 4.76 – 4.51 (m, 1H, -OCH(CH$_3$)$_2$), 3.49 (s, 3H, N$^7$-CH$_3$), 3.05 (s, 3H, N1-CH$_3$), 1.27 (d, J = 7.4 Hz, 6H, -OCH(CH$_3$)$_2$). Anal. Calcd. for C$_{16}$H$_{18}$ClN$_5$O 0.11 CH$_3$(CH$_2$)$_4$CH$_3$: C, 58.61; H, 5.76; Cl, 10.39; N, 20.54. Found: C, 58.36; H, 5.70; Cl, 10.13; N, 20.34.

5-chloro-N-(5-methoxynaphthalen-2-yl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (261)

To a solution of 344 (0.15 g, 0.738 mmol) in acetonitrile (12 mL) was added 5-methoxy-N-methylnaphthalen-2-amine (166 mg, 0.886 mmol) was and the resulting mixture was refluxed for
12 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc to give pale yellow solid 261 (104 mg, 40%). TLC $R_f = 0.50$ (Hexane: EtOAc, 3:1); mp, 178-180.2 °C; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.20 (d, $J = 9.0$ Hz, 1H, Ar), 8.10 (s, 1H, Ar), 7.68 (d, $J = 2.3$ Hz, 1H, Ar), 7.51 (dd, $J = 9.0, 2.3$ Hz, 1H, Ar), 7.43 (d, $J = 7.8$ Hz, 1H, Ar), 7.40 – 7.27 (m, 1H, Ar), 6.98 (d, $J = 7.8$ Hz, 1H, Ar), 3.97 (s, 3H, -OCH$_3$), 3.63 (s, 3H, N$_7$-CH$_3$), 2.92 (s, 3H, N$_1$-CH$_3$). Anal. Calcd. for C$_{18}$H$_{16}$ClN$_5$O: C, 61.11; H, 4.56; Cl, 10.02; N, 19.79. Found: C, 61.19; H, 4.53; Cl, 9.89; N, 19.53.

5-chloro-N,1-dimethyl-N-(4-(methylthio)phenyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine (262)

To a solution of 344 (0.15 g, 0.738 mmol) in acetonitrile (15 mL) was added N-methyl-4-(methylthio)aniline (135.86 mg, 0.886 mmol) and the resulting mixture was refluxed for 12 hours. The solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc to give yellow solid 262 (85 mg, 36%). TLC $R_f = 0.40$ (Hexane: EtOAc, 3:1); mp, 140 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.09 (s, 1H, Ar), 7.31 (d, $J = 8.5$ Hz, 2H, Ar), 7.24 (d, $J = 8.5$ Hz, 2H, Ar), 3.52 (s, 3H, N$_7$-CH$_3$), 3.09 (s, 3H, N$_1$-CH$_3$), 2.49 (s, 3H, -SCH$_3$). Anal. Calcd. for C$_{14}$H$_{14}$ClN$_5$S 0.06 CH$_3$(CH$_2$)$_4$CH$_3$: C, 53.11; H, 4.62; Cl, 10.89; N, 21.52, S, 9.85. Found: C, 53.21; H, 4.52; Cl, 10.81; N, 21.67, S, 9.79.

5-chloro-N-(3-fluoro-4-methoxyphenyl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (263)

To a solution of 344 (0.2 g, 0.985 mmol) in acetonitrile (15 mL) was added 3-fluoro-4-methoxy-N-methylaniline (183.43 mg, 1.18 mmol) and the resulting mixture was refluxed for 12 hours. The
solvent was removed under reduced pressure to obtain a crude solid that was purified via column chromatography eluting with a mixture of hexane and EtOAc (10:1) to give pale yellow solid 263 (146 mg, 46%). TLC $R_f = 0.5$ (Hexane: EtOAc, 3:1); mp, 172-173.7 °C; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.94 (d, $J=3.0$ Hz, 1H, Ar), 6.38-6.26 (m, 2H, Ar), 5.50 (s, 1H, Ar), 3.70 (s, 3H, -OCH$_3$), 3.48 (s, 3H, $N_7$-CH$_3$), 2.61 (s, 3H, $N_1$-CH$_3$). The compound was not sent for biological evaluation and requires further characterization.

2-amino-5-iodonicotinonitrile (353)

To a stirred solution of 2-aminonicotinonitrile 345 (2.0 g, 16.79 mmol) in acetonitrile (15 mL) and acetic acid (10 mL), N-Iodosuccinimide (NIS) (3.78 g, 16.79 mmol) was added. The mixture was heated overnight to 80 °C. Sodium sulfite (4.23 g, 33.58 mmol) was added at room temperature and then the solution was heated to 80°C for 1 h. The crude mixture was diluted in CH$_2$Cl$_2$, then washed with water and the organic layer was basified to pH 10 with NaOH 6N and dried over Na$_2$SO$_4$. The solvent was removed under pressure and the residue was purified by chromatography (CHCl$_3$: MeOH 40:1) to yield 353 as yellow orange solid (2.96 g, 72%). TLC $R_f = 0.76$ (CHCl$_3$: MeOH, 20:1); mp, 99-101.5°C; $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.35 (d, $J = 2.3$ Hz, 1H, Ar), 8.19 (d, $J = 2.3$ Hz, 1H, Ar), 7.11 (s, 2H, exch., -NH$_2$). Anal. Calcd. for C$_{12}$H$_9$N$_3$S: C, 63.41; H, 3.99; N, 18.49; S, 14.11. Found: C, 63.19; H, 4.02; N, 18.47; S, 13.87.

General Procedure for the Synthesis of Compounds 282-289

To a stirred solution of Cs$_2$CO$_3$ (1.1 eq.) in DMF were added 2-amino-5-iodonicotinonitrile (1 eq.), thiols (1.2 eq.), Cul (0.5 eq.) and picolinic acid (0.1 eq.). The mixture was heated 12-24 hours to 100°C and monitored by TLC until reaction was completed. The crude mixture was cooled to room
temperature and copper salts were filtered on celite. The filtrate was diluted in CH₂Cl₂ and washed three times with a saturated solution of LiCl and the organic layer was dried over Na₂SO₄. The solvent was removed under pressure and the residue was purified by chromatography to yield 348 and 354-360 in 58-70% yield. Without further characterization 348 and 354-360, chloroformamidine hydrochloride (1 g), and dimethyl sulfone (4.0 g) were heated in an oil bath at 140 °C under nitrogen for 2-4 hours. The reaction was cooled to room temperature, and water (10 mL) was added slowly to the reaction mixture. The aqueous phase was made basic to pH 10 with ammonium hydroxide, followed by extraction with chloroform (3 × 5 mL). The chloroform extract was dried overnight with Na₂SO₄ and reduced under pressure. The residue was dissolved in a mixture of 50:50 methanol-acetone (v/v), and silica gel was added (3.0 g). After the removal of solvent with a rotary evaporator, the silica gel plug was loaded onto a column and eluted with 10:1 CHCl₃-MeOH (v/v). The fractions containing the required compound were evaporated under reduced pressure to afford the targeted compounds 282-289 in 36-64% yield.

6-(phenylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (282)

To a stirred solution of Cs₂CO₃ (731.36 mg, 2.24 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (500 mg, 2.04 mmol), thiophenol (269.79 mg, 2.45 mmol), picolinic acid (25.12 mg, 0.204 mmol), and copper iodide (194.32 mg, 1.02 mmol). The mixture was heated overnight to 100 °C. General procedure was followed for workup procedure to yield 348 as light orange solid (260 mg, 56%). TLC Rf = 0.36 (hexane: EtOAc, 3:1). Compound 348 was used in the next reaction, without further separation or characterization efforts. Compound 348 (200 mg, 0.879 mmol) was heated at 140 °C with chloroformamidine hydrochloride (405 mg, 3.52 mmol), and dimethyl sulfone (2.0 g) for 2 hours to yield 282 as yellow solid (95 mg, 40% yield). TLC Rf = 0.36 (CHCl₃: MeOH: NH₄OH 20: 1: 1); mp, 174.5-178.2 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.68 (d, J
= 2.4 Hz, 1H, Ar), 8.64 (d, J = 2.4 Hz, 1H, Ar), 7.68 (s, 2H, exch., -NH₂), 7.32 (t, J= 7.3 Hz, 2H, Ar), 7.21 (t, J= 7.3 Hz, 1H, Ar), 7.18 – 7.13 (m, 2H, Ar), 6.65 (s, 2H, exch., -NH₂). Anal. Calcd. for C₁₃H₁₁N₅S 0.24 H₂O: C, 57.04; H, 4.23; N, 25.59, S, 11.71. Found: C, 57.17; H, 4.24; N, 25.38, S, 11.54.

6-((3-methoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (283)

To a stirred solution of Cs₂CO₃ (585.09 mg, 1.80 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (400 mg, 1.63 mmol), 3-methoxybenzenethiol (274.65 mg, 1.96 mmol), picolinic acid (20.10 mg, 0.163 mmol), and copper iodide (155.45 mg, 0.816 mmol). The mixture was heated overnight to 100 °C. General procedure was followed for workup procedure to yield 354 as yellow solid (260 mg, 62%). TLC Rf = 0.63 (hexane: EtOAc, 1:1). Compound 354 was used in the next reaction, without further separation or characterization efforts. Compound 354 (250 mg, 0.971 mmol) was heated at 140 °C with chlorformamidine hydrochloride (446 mg, 3.89 mmol), and dimethyl sulfone (2 g) for 4 hours to yield 283 as a yellow solid (119 mg, 41%). TLC Rf = 0.30 (CHCl₃:MeOH: NH₄OH 10:1:1 ); mp, 167-169.8 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.68 (d, J = 2.6 Hz, 1H, Ar), 8.65 (d, J = 2.6 Hz, 1H, Ar), 7.68 (s, 2H, exch., -NH₂), 7.25-7.20 (m, 1H, Ar), 6.75 (s, 2H, exch., -NH₂), 6.78-6.75 (m, 1H, Ar), 6.70-6.68 (m, 1H, Ar), 6.67-6.65 (m, 1H, Ar), 3.76 (s, 3H, -OCH₃). Anal. Calcd. for C₁₄H₁₃N₅SO 0.52 H₂O: C, 54.46; H, 4.56; N, 22.68; S, 10.38. Found: C, 54.61 H, 4.38; N, 22.30; S, 10.27.

6-((4-methoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (284)

To a stirred solution of Cs₂CO₃ (511.95 mg, 1.57 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (350 mg, 1.43 mmol), 4-methoxybenzenethiol (240.32 mg, 1.71 mmol), picolinic acid (17.6 mg, 0.142 mmol), and copper iodide (136 mg, 0.714 mmol). The mixture was heated overnight to 100°C. General procedure was followed for workup procedure to yield 355 as
yellow solid (235 mg, 64%). TLC $R_f = 0.61$ (hexane: EtOAc, 1:1). Compound 355 was used in the next reaction, without further separation or characterization efforts. Compound 355 (200 mg, 0.777 mmol) was heated at 140 °C with chlorformamidine hydrochloride (357.41 mg, 3.11 mmol), and dimethyl sulfone (2 g) for 4 hours to yield 284 as yellow solid (93 mg, 40%). TLC $R_f = 0.35$ (CHCl$_3$:MeOH: NH$_4$OH 10:1:1 ); mp, 170-172 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.26 (d, $J= 2.4$ Hz, 1H, Ar), 8.02 (d, $J= 2.4$ Hz, 1H, Ar), 7.65 (s, 2H, exch., -NH$_2$), 7.27 (d, $J= 8.8$ Hz, 2H, Ar), 6.93 (d, $J= 8.8$ Hz, 2H, Ar), 6.59 (s, 2H, exch., -NH$_2$), 3.80 (s, 3H, -OCH$_3$). Anal. Calcd. for C$_{14}$H$_{13}$N$_5$OS 0.09 CHCl$_3$: C, 54.58; H, 4.26; N, 22.59; S, 10.34. Found: C, 54.56 H, 4.40; N, 22.60; S, 10.34.

6-((3,4-dimethoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (285)

To a stirred solution of Cs$_2$CO$_3$ (585.09 mg, 1.80 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (400 mg, 1.63 mmol), 3,4-dimethoxybenzenethiol (333.47 mg, 1.96 mmol), picolinic acid (20.10 mg, 0.163 mmol), and copper iodide (155.45 mg, 0.816 mmol). The mixture was heated overnight to 100°C. General procedure was followed for workup procedure to yield 356 as yellow solid (272 mg, 58%). TLC $R_f = 0.45$ (hexane: EtOAc, 1:1). Compound 356 was used in the next reaction, without further separation or characterization efforts. Compound 356 (220 mg, 0.765 mmol) was heated at 140 °C with chlorformamidine hydrochloride (352 mg, 3.06 mmol), and dimethyl sulfone (2 g) for 4 hours to yield 285 as yellow solid (90 mg, 36%). TLC $R_f = 0.55$ (CHCl$_3$:MeOH: NH$_4$OH 10:1:1 ); mp, 186 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.65-8.59 (m, 2H, Ar), 7.66 (s, 2H, exch., -NH$_2$), 7.02-6.92 (m, 2H, Ar), 6.87-6.78 (m, 1H, Ar), 6.59 (s, 2H, exch., -NH$_2$), 3.74 (s, 6H, -OCH$_3$). Anal. Calcd. for C$_{15}$H$_{15}$N$_5$O$_2$S 0.17 CHCl$_3$: C, 52.05; H, 4.37; N, 20.00; S, 9.16. Found: C, 52.06; H, 4.41; N, 19.96; S, 9.17.
6-((4-fluorophenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (286)

To a stirred solution of Cs$_2$CO$_3$ (511.95 mg, 1.57 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (350 mg, 1.43 mmol), 4-fluorobenzenethiol (219.69 mg, 1.71 mmol), picolinic acid (17.6 mg, 0.143 mmol), and copper iodide (136 mg, 0.714 mmol). The mixture was heated overnight to 100°C. General procedure was followed for workup procedure to yield 357 as yellow solid (227 mg, 65%). TLC $R_f = 0.34$ (hexane: EtOAc, 3:1). Compound 357 was used in the next reaction, without further separation or characterization efforts. Compound 357 (200 mg, 0.815 mmol) was heated at 140 °C with chlorformamidine hydrochloride (374.95 mg, 3.26 mmol), and dimethyl sulfone (2 g) for 4 hours to yield 286 as yellow solid (138 mg, 59%). TLC $R_f = 0.22$ (CHCl$_3$: MeOH: NH$_4$OH 20:1:1); mp, 189.2-192.1 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.72 (d, $J = 2.6$ Hz, 1H, Ar), 8.62 (d, $J = 2.6$ Hz, 1H, Ar), 7.73 (s, 2H, exch., -NH$_2$), 7.29 – 7.25 (m, 2H, Ar), 7.20-7.16 (m, 2H, Ar), 6.62 (s, 2H, exch., -NH$_2$). Anal. Caled. for C$_{13}$H$_{10}$FN$_5$S 0.03 CHCl$_3$: C, 53.72; H, 3.47; F, 6.52; N, 24.03; S, 11.00. Found: C, 53.83; H, 3.48; F, 6.35; N, 23.85; S, 11.03.

6-((4-(trifluoromethoxy)phenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (287)

To a stirred solution of Cs$_2$CO$_3$ (585 mg, 1.80 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (400 mg, 1.63 mmol), 4-(trifluoromethoxy)benzenethiol (380.38 mg, 1.96 mmol), picolinic acid (20.10 mg, 0.163 mmol), and copper iodide (155.45 mg, 0.816 mmol). The mixture was heated overnight to 100°C. General procedure was followed for workup procedure to yield 358 as pale-yellow solid (350 mg, 69%). TLC $R_f = 0.32$ (hexane: EtOAc, 3:1). Compound 358 was used in the next reaction, without further separation or characterization efforts. Compound 358 (150 mg, 0.481 mmol) was heated at 140 °C with chlorformamidine hydrochloride (221.58 mg, 1.93 mmol), and dimethyl sulfone (2.0 g) for 4 hours to yield 287 as a light yellow solid (108 mg, 64%). TLC $R_f = 0.63$ (CHCl$_3$: MeOH: NH$_4$OH 20:1:1); mp, 191 °C; $^1$H NMR (400 MHz,
DMSO-$d_6$ δ 8.70 (d, $J = 2.5$ Hz, 1H, Ar), 8.67 (d, $J = 2.5$ Hz, 1H, Ar), 7.68 (s, 2H, exch., -NH$_2$), 7.33 (d, $J = 8.5$ Hz, 2H, Ar), 7.26 (d, $J = 8.5$ Hz, 2H, Ar), 6.67 (s, 2H, exch., -NH$_2$). Anal. Calcd. for C$_{14}$H$_{10}$F$_3$N$_5$O$_2$ 0.17 CH$_3$OH 0.06 HCl: C, 47.11; H, 3.00; F, 15.77; N, 19.38, S, 8.87. Found: C, 47.11; H, 3.02; F, 15.78; N, 19.38, S, 8.89.

6-(naphthalen-1-ylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (288)

To a stirred solution of Cs$_2$CO$_3$ (365.68 mg, 1.12 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (250 mg, 1.02 mmol), naphthalene-1-thiol (196.19 mg, 1.22 mmol), picolinic acid (12.56 mg, 0.102 mmol), and copper iodide (97.16 mg, 0.510 mmol). The mixture was heated overnight to 100°C. General procedure was followed for workup procedure to yield 359 as off-white solid (198 mg, 70%). TLC $R_f = 0.4$ (hexane: EtOAc, 3:1). Compound 359 was used in the next reaction, without further separation or characterization efforts. Compound 359 (150 mg, 0.504 mmol) was heated at 140 °C with chlorformamidine hydrochloride (249 mg, 2.16 mmol), and dimethyl sulfone (2.0 g) for 4 hours to yield 288 as off-white solid (105 mg, 61%). TLC $R_f = 0.20$ (CHCl$_3$: MeOH: NH$_4$OH 20:1:1); mp, 226.5-228 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.70 (d, $J = 2.3$ Hz, 1H, Ar), 8.63 (d, $J = 2.3$ Hz, 1H, Ar), 8.33 (d, $J = 8.3$ Hz, 1H, Ar), 8.06 – 7.94 (m, 1H, Ar), 7.85 (d, $J = 8.3$ Hz, 1H, Ar), 7.69 – 7.64 (m, 2H, Ar), 7.61 (s, 2H, exch., -NH$_2$), 7.44 (t, $J = 7.8$ Hz, 1H, Ar), 7.30 – 7.13 (m, 1H, Ar), 6.62 (s, 2H, exch., -NH$_2$). Anal. Calcd. for C$_{17}$H$_{13}$N$_5$S 0.68 CH$_3$OH: C, 62.25; H, 4.64; N, 20.54; S, 9.40. Found: C, 61.98; H, 4.35; N, 20.70; S, 9.49.

6-(naphthalen-2-ylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (289)

To a stirred solution of Cs$_2$CO$_3$ (321.80 mg, 0.987 mmol) in DMF were added 2-amino-5-iodonicotinonitrile (220 mg, 0.897 mmol), naphthalene-2-thiol (172.64 mg, 1.08 mmol), picolinic acid (11.05 mg, 0.089 mmol), and copper iodide (85.5 mg, 0.449 mmol). The mixture was heated
overnight to 100°C. General procedure was followed for workup procedure to yield 360 as off-white solid (169 mg, 68%). TLC $R_f = 0.41$ (hexane: EtOAc, 3:1). Compound 360 was used in the next reaction, without further separation or characterization efforts. Compound 360 (120 mg, 0.432 mmol) was heated at 140 °C with chlorformamidine hydrochloride (200 mg, 1.74 mmol), and dimethyl sulfone (2.0 g) for 4 hours to yield 289 as off-white solid (82 mg, 60%). TLC $R_f = 0.18$ (CHCl$_3$: MeOH: NH$_4$OH 20:1:1); mp, 220-221.8 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.72 (d, $J = 2.3$ Hz, 1H, Ar), 8.70 (d, $J = 2.3$ Hz, 1H, Ar) 7.88 (d, $J = 8.7$ Hz, 2H, Ar), 7.84 – 7.77 (m, 1H, Ar), 7.69 (s, 2H, exch., -NH$_2$), 7.66 (d, $J = 1.9$ Hz, 1H, Ar), 7.53 – 7.44 (m, 2H, Ar), 7.32 (dd, $J = 8.7, 1.9$ Hz, 1H, Ar), 6.66 (s, 2H, exch., -NH$_2$). Anal. Calcd. for C$_{17}$H$_{13}$N$_5$S 0.06 CHCl$_3$: C, 62.75; H, 4.03; N, 21.45; S, 9.82. Found: C, 62.70; H, 4.22; N, 21.55; S, 9.82.

**General procedure for synthesis of 364-365**

In a 20 mL vial for microwave reaction were added a mixture of palladium chloride triphenylphosphine, triethylamine, methyl 4-iodobenzoate 361, and anhydrous acetonitrile. To the stirred mixture were added copper(I) iodide and appropriate alkyne alcohols 362-363 and the vial was sealed and put into the microwave reactor at 100 °C for 10 min. Silica gel (5 g) was added, and the solvent was evaporated under reduced pressure. The resulting plug was loaded on to a silica gel column and eluted with Hexane followed by 20% EtOAc in Hexane. The desired fraction (TLC) was collected, and the solvent was evaporated under reduced pressure to afford the target compounds.
Methyl 4-(3-hydroxyprop-1-yn-1-yl)benzoate (364)

Compound 364 was synthesized using the general method described for the preparation of 364-365. Methyl 4-iodobenzoate 361 (2.8 g, 10.69 mmol) in a solution of acetonitrile (5 mL) in a microwave reactor (10 mL) were mixed with PdCl$_2$ (75.8 mg, 0.427 mmol) and Ph$_3$P (112.10 mg, 0.427 mmol) and stirred for 10 mins. Copper iodide (325.6 mg, 1.71 mmol), propargyl alcohol 362 (0.67 mL, 11.75 mmol) and Et$_3$N (4.5 mL, 32.06 mmol) were added to the microwave vial and reacted for 1h at 100 $^\circ$C to give 364 as a yellow solid (1.83 g, 84%). TLC $R_f = 0.36$ (Hexane: EtOAc, 3:1); mp, 90-91.6 $^\circ$C; $^1$H-NMR (400 MHz) (Me$_2$SO-$d_6$) $\delta$ 7.95 (d, $J = 8.4$ Hz, 2H, Ar), 7.57 (d, $J = 8.4$ Hz, 2H, Ar), 5.42 (s,1H, exch., -OH), 4.34 (s, 2H, -CH$_2$-), 3.86 (s, 3 H, -OCH$_3$). The $^1$H-NMR matched the $^1$H-NMR reported in the literature.$^{230}$ Anal. Calcd. for C$_{11}$H$_{10}$O$_3$: C, 69.46; H, 5.30. Found: C, 69.43; H, 5.28.

Methyl 4-(4-hydroxybut-1-yn-1-yl)benzoate (365)

Compound 365 was synthesized using the general method described for the preparation of 364-365. Methyl 4-iodobenzoate 361 (2.5 g, 9.54 mmol) in a solution of acetonitrile in a microwave reactor (10 mL) were mixed with PdCl$_2$ (67.6 mg, 0.381 mmol) and Ph$_3$P (100.09 mg, 0.381 mmol) stirred for 10 mins. Copper iodide (290.7 mg, 1.53 mmol), but-3-yn-1-ol (0.8 mL, 10.49 mmol) and Et$_3$N ( 4.0 mL, 28.62 mmol) were added to the microwave vial and reacted for 1 hour at 100 $^\circ$C to give 365 as a yellow solid (1.68 g, 86%); TLC $R_f = 0.45$ (Hexane: EtOAc, 3:1); mp, 92-94.8 $^\circ$C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.87 (d, $J = 8.3$ Hz, 2H, Ar), 7.42 (d, $J = 8.3$ Hz, 2H, Ar), 4.44 (t, $J = 4.79$ Hz, 1H, exch., -OH), 3.83 (s, 3H, -OCH$_3$), 2.64 (t, $J = 7.7$ Hz, 2H, -CH$_2$-), 1.67 – 1.54 (m, 2H, -CH$_2$-). The $^1$H-NMR matched the $^1$H-NMR reported in the literature.$^{230}$ Anal. Calcd. for C$_{12}$H$_{12}$O$_3$: C, 70.58; H, 5.92. Found: C, 70.31; H, 5.95.
**General procedure for synthesis of 366-367**

To a Parr flask was added 364-365 10% palladium on activated carbon (50% w/w), and MeOH (100 mL). Hydrogenation was carried out at 55 psi of H\(_2\) for 12 h. The reaction mixture was filtered through Celite, washed with MeOH (100 mL), and concentrated under reduced pressure to give crude mixture containing 366-367 as colorless liquid. Without chromatographic separation, these compounds were used for the next reaction.

**Methyl 4-(3-hydroxypropyl)benzoate (366)**

Compound 366 was prepared using the general method described, from 364 (1.60 g, 8.41 mmol) to give 1.32 g (81% yield) of 366 as a clear oil; TLC \(R_f = 0.15\) (Hexane: EtOAc, 3:1); \(^1\)H-NMR (400 MHz) (Me\(_2\)SO-\(d_6\)) 7.87 (d, \(J = 8.0\) Hz, 2H, Ar), 7.35 (d, \(J = 8.0\) Hz, 2H, Ar), 4.52 (s, 1H, exch., -OH), 3.82 (s, 3 H, -OCH\(_3\)), 3.42 (t, \(J = 6.44\) Hz, 2H, -CH\(_2\)-), 2.71-2.65 (m, 2H, -CH\(_2\)-), 1.76-1.70 (m, 2H, -CH\(_2\)-). This compound was used for the next reaction without further characterization.

**Methyl 4-(4-hydroxybutyl)benzoate (367)**

Compound 367 was prepared using the general method described, from 365 (1.60 g, 7.83 mmol) to give 1.26 g (83%) of 367 as a clear oil; TLC \(R_f = 0.20\) (Hexane: EtOAc, 3:1); \(^1\)H-NMR (400 MHz) (Me\(_2\)SO-\(d_6\)) 7.94 (d, \(J = 8.0\) Hz, 2H, Ar), 7.56 (d, \(J = 8.0\) Hz, 2H, Ar), 4.41 (t, \(J = 4.79\) Hz, 1H, exch., -OH), 3.85 (s, 3 H, -OCH\(_3\)), 3.44-3.38 (m, 2H, -CH\(_2\)-), 2.65 (t, \(J = 7.7\) Hz, 2H, -CH\(_2\)-), 1.66-1.56 (m, 2H, -CH\(_2\)-), 1.46-1.38 (m, 2H, -CH\(_2\)-). This compound was used for the next reaction without further characterization.
General procedure for synthesis of 368-369
To the alcohols 366-367 was added triethylamine (1 equivalent) and dichloromethane (25 mL). The reaction was cooled to 0 °C and purged with nitrogen gas. Under anhydrous conditions, methanesulfonyl chloride (1.05 equivalent) was added dropwise over 30 minutes. The reaction was stirred at room temperature for 2 hours and the reaction was added into sodium bicarbonate solution (25 mL). The water layer was washed thrice with dichloromethane (100 mL). The dichloromethane was evaporated to obtain a colorless liquid product. To the intermediate in acetone, sodium iodide (1 equivalent) was added and refluxed for 8 hours. The reaction mixture was filtered. The filtrate was evaporated to obtain 368-369.

Methyl 4-(3-iodopropyl)benzoate (368)
Compound 368 was prepared using the general method described from 366 (1 g, 5.15 mmol), methanesulfonyl chloride (0.8 mL, 10.30 mmol) and triethylamine (1.65 mL, 11.84 mmol) to form the intermediate. To this solution mixture sodium iodide (2.89 g, 19.28 mmol) was added and the procedure was followed to give 1.0 g (64% yield in two steps) of 368 as a clear oil; TLC $R_f = 0.60$ (Hexane: EtOAc, 2:1); $^1$H-NMR (400 MHz) (Me$_2$SO-$d_6$) δ 7.90 (d, $J = 8.0$ Hz, 2H, Ar), 7.36 (d, $J = 8.0$ Hz, 2H, Ar), 3.84 (s, 3 H, -OCH$_3$), 3.24 (t, $J = 6.4$ Hz, 2H, -CH$_2$-), 2.73 (t, $J = 7.5$ Hz, 2H, -CH$_2$-), 2.11-2.07 (m, 2H, -CH$_2$-). This compound was used for the next reaction without further characterization.

Methyl 4-(4-iodobutyl)benzoate (369)
Compound 369 was prepared using the general method described from 367 (1 g, 4.8 mmol), methanesulfonyl chloride (0.74 mL, 9.60 mmol) and triethylamine (1.54 mL, 11.04 mmol) to form the intermediate. To this sodium iodide (2.52 g, 16.80 mmol) was added and the procedure was
followed to give 0.9 g (60% in two steps) of 369 as a clear oil; TLC $R_f = 0.50$ (Hexane: EtOAc, 2:1); $^1$H-NMR (500 MHz) ($\text{Me}_2\text{SO-d6}$) $\delta$ 7.88 (d, $J = 8.2$ Hz, 2H, Ar), 7.34 (d, $J = 8.2$ Hz, 2H, Ar), 3.83 (s, 3H, -OCH$_3$), 3.30 (t, $J = 6.7$ Hz, 2H, -CH$_2$-), 2.68 (t, $J = 7.5$ Hz, 2H, -CH$_2$-), 1.81 – 1.73 (m, 2H, -CH$_2$-), 1.72-1.64 (m, 2H, -CH$_2$). This compound was used for the next reaction without further characterization.

**General procedure for synthesis of 370-373**

In a solution of DMF or acetonitrile, Methyl 4-nitro-$1H$-pyrazole-5-carboxylate 374 was alkylated using K$_2$CO$_3$ or Cs$_2$CO$_3$ with compound 368 and 369, respectively. The reaction was stirred for 12 hours at room temperature. The solvent was removed under vacuum and a silica gel plug was made, and the crude material was purified by column chromatography using chloroform methanol system to give 370-373. Structure of compound 371 was confirmed by x-ray crystallography which finally allowed full assignment of the structures for both regioisomers 370 and 371.

**Methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-$1H$-pyrazole-5-carboxylate (372)**

Compound 372 was prepared using the general method described from 374 (2.0 g, 11.69 mmol) and 368 (9.48 g, 31.17 mmol). A flash column chromatographic separation was performed using chloroform methanol as eluent to afford intermediate 372 (1.18 g, 35% yield) as a white semi-solid.; TLC $R_f = 0.27$ (CHCl$_3$: MeOH: NH$_4$OH, 20:1:0.05); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.42 (s, 1H, Ar), 7.89 (d, $J = 8.20$ Hz, 2H, Ar), 7.36 (d, $J = 8.20$ Hz, 2H, Ar), 4.29 (t, $J = 6.6$ Hz, 2H, -CH$_2$-), 3.94 (s, 3H, -OCH$_3$), 3.84 (s, 3H, -OCH$_3$), 2.67 (t, $J = 7.5$ Hz, 2H, -CH$_2$-), 2.20 – 2.11 (m, 2H, -CH$_2$-). This compound was used for the next reaction without further characterization.
Methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-1\textit{H}-pyrazole-3-carboxylate (373)

Compound 373 was prepared using the general method described from 374 (2.0 g, 11.69 mmol) and 368 (9.48 g, 31.17 mmol). A flash column chromatographic separation was performed using chloroform methanol as eluent to afford intermediate 373 (2.23 g, 66% yield). TLC $R_f = 0.12$ (CHCl$_3$: MeOH: NH$_4$OH, 20:1:0.05); mp, 112-114 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.00 (s, 1H, Ar), 7.87 (d, $J = 8.30$ Hz, 2H, Ar), 7.35 (d, $J = 8.30$ Hz, 2H, Ar), 4.24 (t, $J = 7.0$ Hz, 2H, -CH$_2$), 3.88 (s, 2H, -OCH$_3$), 3.83 (s, 3H, -OCH$_3$), 2.67 (t, $J = 7.5$ Hz, 2H, -CH$_2$), 2.21 – 2.14 (p, $J = 7.5$ Hz, 2H, -CH$_2$). Anal. Calcd. for C$_{17}$H$_{19}$N$_3$O$_6$: C, 70.58; H, 5.92. Found: C, 70.31; H, 5.95.

Methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1\textit{H}-pyrazole-5-carboxylate (370)

Compound 370 was prepared using the general method described from 374 (2.5 g, 14.61 mmol) and 369 (12.40 g, 38.96 mmol). A flash column chromatographic separation was performed using chloroform methanol as eluent to afford intermediate 370 (1.4 g, 32% yield) as a white semi-solid. TLC $R_f = 0.32$ (CHCl$_3$: MeOH: NH$_4$OH, 20:1:0.05); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.41 (s, 1H, Ar), 7.87 (d, $J = 8.0$ Hz, 2H, Ar), 7.33 (d, $J = 8.0$ Hz, 2H, Ar), 4.28 (t, $J = 7.0$ Hz, 2H, -CH$_2$), 3.86 (s, 3H, -OCH$_3$), 3.83 (s, 3H, -OCH$_3$), 2.67 (t, $J = 7.6$ Hz, 2H, -CH$_2$), 2.18 (p, $J = 7.4$ Hz, 2H, -CH$_2$), 1.56 (p, $J = 7.4$ Hz, 2H, -CH$_2$). This compound was used for the next reaction without further characterization.

Methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1\textit{H}-pyrazole-3-carboxylate (371)

Compound 371 was prepared using the general method described from 374 (2.5 g, 14.61 mmol) and 369 (12.40 g, 38.96 mmol). A flash column chromatographic separation was performed using chloroform methanol as eluent to afford intermediate 371 (2.8 g, 64% yield); TLC $R_f = 0.16$
(CHCl₃: MeOH, 20:1); mp, 136-137.8 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 9.02 (s, 1H, Ar), 7.88 (d, J = 8.0 Hz, 2H, Ar), 7.34 (d, J = 8.0 Hz, 2H, Ar), 4.24 (t, J = 6.8 Hz, 2H, -CH₂-), 3.88 (s, 3H, -OCH₃), 3.84 (s, 3H, -OCH₃), 2.65 (t, J = 7.5 Hz, 2H, -CH₂-), 1.83 (p, J = 7.0 Hz, 2H, -CH₂-), 1.56 (t, J = 7.5 Hz, 2H, -CH₂-). Anal. Calcd. for C₁₇H₁₉N₃O₆ 0.047 CHCl₃: C, 55.79; H, 5.23; N, 11.45. Found: C, 55.82; H, 5.38; N, 11.28.

**General procedure for synthesis of 375-376 and 381-382**

Compounds **375-376** and **381-382** were generated using general method. To a Parr flask was added **370-373**, 10% palladium on activated carbon (50% w/w), and MeOH (100 mL). Hydrogenation was carried out at 40 psi of H₂ for 30 min. The reaction mixture was filtered through Celite, washed with MeOH (40 mL) and chloroform (20 mL), and concentrated under reduced pressure to give crude mixture containing **375-376** and **381-382**. Without chromatographic separation, these compounds were used for the next reaction.

**Methyl 4-amino-1-(3-(4-(methoxycarbonyl)phenyl)propyl)-1H-pyrazole-5-carboxylate (375)**

Compound **375** was prepared using the general method described from **372** (1.0 g, 2.88 mmol) by catalytic reduction by H₂ in MeOH at 40 psi. Intermediate **375** appeared as a yellow liquid (676 mg, 74% yield). TLC Rf = 0.25 (CHCl₃: MeOH, 10:1); ¹H NMR (400 MHz, DMSO-d₆) δ 7.87 (d, J = 8.3 Hz, 2H, Ar), 7.34 (d, J = 8.3 Hz, 2H, Ar), 7.05 (s, 1H, Ar), 5.04 (s, br, 2H, exch., -NH₂), 4.30 (t, J = 7.0 Hz, 2H, -CH₂-), 3.83 (s, 3H, -OCH₃), 3.76 (s, 3H, -OCH₃), 2.59 (t, J = 7.7 Hz, 2H, -CH₂-), 2.01 – 1.86 (m, 2H, -CH₂-). This compound was used for the next reaction without further characterization.
Methyl 4-amino-1-(3-(4-(methoxycarbonyl)phenyl)propyl)-1H-pyrazole-3-carboxylate (381)

Compound 381 was prepared using the general method described from 373 (2.0 g, 5.76 mmol) by catalytic reduction by H\textsubscript{2} in MeOH at 40 psi. Intermediate 381 appeared as a yellow liquid (1.33 g, 73% yield); TLC \emph{Rf} = 0.24 (CHCl\textsubscript{3}: MeOH, 10:1); \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \emph{δ} 7.85 (d, \emph{J} = 8.4 Hz, 2H, Ar), 7.33 (d, \emph{J} = 8.4 Hz, 2H, Ar), 7.62 (s, 1H, Ar), 5.01 (s, br, 2H, exch., -NH\textsubscript{2}), 4.26 (t, \emph{J} = 7.0 Hz, 2H, -CH\textsubscript{2}-), 3.81 (s, 3H, -OCH\textsubscript{3}), 3.75 (s, 3H, -OCH\textsubscript{3}), 2.58 (t, \emph{J} = 7.6 Hz, 2H, -CH\textsubscript{2}-), 2.00 – 1.85 (m, 2H, -CH\textsubscript{2}-). This compound was used for the next reaction without further characterization.

Methyl 4-amino-1-(4-(4-(methoxycarbonyl)phenyl)butyl)-1H-pyrazole-5-carboxylate (376)

Compound 376 was prepared using the general method described from 370 (1.2 g, 3.32 mmol) by catalytic reduction with H\textsubscript{2} in MeOH at 40 psi. Intermediate 376 appeared as a yellow liquid (858 mg, 78% yield); TLC \emph{Rf} = 0.32 (CHCl\textsubscript{3}: MeOH, 10:1); \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}\textsubscript{6}) \emph{δ} 7.87 (d, \emph{J} = 8.3, 2H, Ar), 7.29 (d, \emph{J} = 8.3, 2H, Ar), 7.02 (s, 1H, Ar), 4.96 (s, br, 2H, exch., -NH\textsubscript{2}), 4.30 (t, \emph{J} = 6.9 Hz, 2H, -CH\textsubscript{2}-), 3.82 (s, 3H, -OCH\textsubscript{3}), 3.77 (s, 3H, -OCH\textsubscript{3}), 2.64 (t, \emph{J} = 7.5 Hz, 2H, -CH\textsubscript{2}-), 1.72-1.60 (m, 2H, -CH\textsubscript{2}-), 1.54-1.41 (m, 2H, -CH\textsubscript{2}-). This compound was used for the next reaction without further characterization.

Methyl 4-amino-1-(4-(4-(methoxycarbonyl)phenyl)butyl)-1H-pyrazole-3-carboxylate (382)

Compound 382 was prepared using the general method described from 371 (2.5 g, 6.92 mmol) by catalytic reduction with H\textsubscript{2} in MeOH at 40 psi. Intermediate 382 appeared as a yellow liquid (1.74 g, 76% yield). TLC \emph{Rf} = 0.31 (CHCl\textsubscript{3}: MeOH, 10:1); \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \emph{δ} 7.87 (d,
$J = 8.2 \text{ Hz, } 2\text{H, Ar}$), 7.33 (d, $J = 8.2 \text{ Hz, } 2\text{H, Ar}$), 7.17 (s, 1H, Ar), 4.70 (s, br, 2H, exch., -NH$_2$), 4.03 (t, $J = 7.0 \text{ Hz, } 2\text{H, -CH}_2-$), 3.83 (s, 3H, -OCH$_3$), 3.75 (s, 3H, -OCH$_3$), 2.65 (t, $J = 7.7 \text{ Hz, } 2\text{H, -CH}_2-$), 1.74 (p, $J = 7.6 \text{ Hz, } 2\text{H, -CH}_2-$), 1.51 (p, $J = 7.0 \text{ Hz, } 2\text{H, -CH}_2-$). Anal. Calcd. for C$_{17}$H$_{21}$N$_3$O$_4$ 0.35 CHCl$_3$: C, 55.75; H, 5.76; N, 11.25. Found: C, 55.72; H, 6.04; N, 11.43.

**General procedure for synthesis of 377-378 and 383-384.**

Compounds 377-378 and 383-384 were dissolved in MeOH (10 mL), and 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea (3.3 mmol) was added followed by AcOH (15 mmol). The mixture was stirred at room temperature overnight and became a thick paste. NaOMe in MeOH (25%) (22 mmol) was added, and stirring was continued at room temperature overnight. Column chromatography was performed to finally get the compounds 378 and 383-384. Compound 377 was not characterized and requires further characterization.

Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoate (378)

Compound 378 was prepared using the general method described from 376 (400 mg, 1.21 mmol) and 375 appeared as yellow solid (255.8 mg, 48% yield in two steps); TLC $R_f = 0.41$ (CHCl$_3$: MeOH, 20:1); mp, 222-224.6 $^\circ\text{C}$; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.56 (s, br, 1H, exch., -NH), 10.81 (s, br, 1H, exch., -NH), 7.88 (d, $J=8.2 \text{ Hz, } 2\text{H, Ar}$), 7.80 (s, 1H, Ar), 7.31 (d, $J=8.2 \text{ Hz, } 2\text{H, Ar}$), 4.31 (t, $J = 6.9 \text{ Hz, } 2\text{H, -CH}_2-$), 2.63 (t, $J = 7.5 \text{ Hz, } 2\text{H, -CH}_2-$), 3.82 (s, 3H, -OCH$_3$), 1.86-1.77 (m, 2H, -CH$_2-$), 1.69-1.60 (m, 2H, -CH$_2-$), 1.49 (s, 9H, -CH$_3$). Anal. Calcd. for C$_{22}$H$_{27}$N$_5$O$_5$ 0.10 CH$_3$(CH$_2$)$_4$CH$_3$ 0.23 CH$_3$COOH: C, 59.70; H, 6.37; N, 15.08. Found: C, 59.71; H, 6.33; N, 15.07.
Methyl 4-(4-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (383)

Compound 383 was prepared using the general method described from 381 (1 g, 3.15 mmol) and 383 appeared as light yellow solid (565.7 mg, 42% yield in two steps); TLC Rf = 0.38 (CHCl₃: MeOH, 20:1); mp, 210-212.1°C; ¹H NMR (500 MHz, DMSO-d₆) δ 7.96 (s, 1H, Ar), 7.88 (d, J = 8.3 Hz, 2H, Ar), 7.37 (d, J = 8.3 Hz, 2H, Ar), 4.27 (t, J = 6.9 Hz, 2H, -CH₂-), 3.84 (s, 3H, -OCH₃), 2.64 (t, J = 7.5 Hz, 2H, -CH₂-), 2.22-2.12 (m, 2H, -CH₂-), 1.68 (s, 9H, -CH₃). This compound was used for the next reaction without further characterization.

Methyl 4-(4-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (384)

Compound 384 was prepared using the general method described from 382 (800 mg, 2.41 mmol) and 384 appeared as yellow solid (522.2 mg, 49% yield in two steps); TLC Rf = 0.46 (CHCl₃: MeOH, 20:1); mp, 218-219 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 11.30 (s, br, 1H, exch., -NH), 10.68 (s, br, 1H, exch., -NH), 8.15 (s, 1H, Ar), 7.87 (d, J=8.20 Hz, 2H, Ar), 7.34 (d, J=8.20 Hz, 2H, Ar), 4.32 (t, J = 6.9 Hz, 2H, -CH₂-), 3.83 (s, 3H, -OCH₃), 2.69 (t, J = 7.5 Hz, 2H, -CH₂-), 1.87 (p, J = 7.2 Hz, 2H, -CH₂-), 1.49 (p, J = 7.9 Hz, 2H, -CH₂-), 1.51 (s, 9H, -CH₃). Anal. Calcd. for C₂₂H₂₇N₅O₅ 0.97 CH₃COOH: C, 57.53; H, 6.23; N, 14.01. Found: C, 57.46; H, 6.30; N, 14.10.

General procedure for synthesis of 379-380 and 385-386

The compounds of 379-380 and 385-386 from previous steps was added to 1 N NaOH (2 mL), and the mixture was heated at 55 °C for 3 h. The mixture was cooled and acidified using 1 N hydrochloric acid. The precipitate was collected and dried under. These acids were used for the
next reaction without further characterization where in anhydrous DMF (10 mL) with the acid intermediates were added \( N \)-methylmorpholine and 2-chloro-4,6-dimethoxy-1,3,5-triazine. The resulting mixtures were stirred at room temperature for 2 h. To this mixture were added \( N \)-methylmorpholine and \( L \)-glutamate diethyl ester hydrochloride. The reaction mixtures were stirred for an additional 4 h at room temperature. Silica gel (400 mg) was then added, and the solvent was evaporated under reduced pressure. The resulting plug was loaded on to a silica gel column with 5% MeOH in CHCl\(_3\) as the eluent. Fractions that showed the desired spot (TLC) were pooled and the solvent evaporated to dryness to afford compounds 380 and 385-386. Compound 379 was not characterized and requires further characterization.

**Diethyl (4-(4-(5-amino-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoyl)-\( L \)-glutamate (380)**

Crude acid (220 mg, 0.672 mmol), were used for the next reaction without further characterization where in anhydrous DMF (10 mL) with the acid intermediates were added \( N \)-methylmorpholine (0.13 mL, 1.21 mmol), and 2-chloro-4,6-dimethoxy-1,3,5-triazine (212.40 mg, 1.21 mmol). The resulting mixtures were stirred at room temperature for 2 h. To this mixture were added \( N \)-methylmorpholine (0.13 mL, 1.21 mmol), and \( L \)-glutamate diethyl ester hydrochloride (191.23 mg, 0.940 mmol). The reaction mixtures were stirred for an additional 4 h at room temperature to get 380 in 68% yield (234.25 mg); mp: 238-240.1 °C; \(^1\)H NMR (400 MHz, DMSO-\( d_6\)) \( \delta \) 10.92 (s, 1H, exch., -NH), 8.66 (d, \( J = 7.5 \) Hz, 1H, exch., -NH), 7.78 (d, \( J = 8.1 \) Hz, 2H, Ar), 7.50 (s, 1H, Ar), 7.26 (d, \( J = 8.1 \) Hz, 2H, Ar), 6.04 (s, 2H, exch., -NH\(_2\)), 4.43-4.39 (m, 1H, \( \alpha \)-CH), 4.45 (t, \( J = 6.9 \) Hz, 2H, -CH\(_2\)-), 4.14 – 4.01 (m, 4H, -COOCH\(_2\)-CH\(_3\)), 2.63 (t, \( J = 7.5 \) Hz, 2H, -CH\(_2\)-), 2.44 (t, \( J = 7.5 \) Hz, 2H, \( \gamma \)-CH\(_2\)-), 2.13-1.97 (m, 2H, \( \beta \)-CH\(_2\)-), 1.78 (p, \( J = 7.5 \) Hz, 2H, -CH\(_2\)-), 1.49 (p, \( J = 7.9\)
Hz, 2H, -CH₂-), 1.26-1.12 (m, 6H, -COOC₂H₅). Anal. Calcd. for C₂₅H₃₂N₆O₆ 0.57 CH₃OH: C, 57.85; H, 6.51; N, 15.83. Found: C, 57.52; H, 6.47; N, 16.22.

**Methyl 4-(4-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (385)**

Crude acid (250 mg, 0.797 mmol), were used for the next reaction without further characterization where in anhydrous DMF (10 mL) with the acid intermediates were added N-methylmorpholine (0.16 mL, 1.44 mmol), and 2-chloro-4,6-dimethoxy-1,3,5-triazine (252.6 mg, 1.44 mmol). The resulting mixtures were stirred at room temperature for 2 h. To this mixture were added N-methylmorpholine (0.16 mL, 1.44 mmol), and L-glutamate diethyl ester hydrochloride (227.03 mg, 1.21 mmol). The reaction mixtures were stirred for an additional 4 h at room temperature to get 385 in 62% yield (246.6 mg); mp: 228-230 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 10.55 (s, 1H, exch., -NH), 8.68 (d, J = 7.5 Hz, 1H, exch., -NH), 7.88 (d, J = 8.2 Hz, 2H, Ar), 7.79 (s, 1H, Ar), 7.34 (d, J = 8.2 Hz, 2H, Ar), 5.90 (s, 2H, exch., -NH₂), 4.44-4.38 (m, 1H, α-CH), 4.23 (t, J = 6.9 Hz, 2H, -CH₂-), 4.13-4.01 (m, 4H, -OCH₂CH₃), 2.68-2.64 (m, 2H, -CH₂-), 2.61-2.55 (m, 2H, -CH₂-), 2.44 (t, J = 7.6 Hz, 2H, γ-CH₂-), 2.20-2.13 (m, 2H, β-CH₂-), 1.24-1.15 (m, 6H, -OCH₂CH₃).

MS (ESI) m/z calculated for C₂₄H₃₀N₆O₆ [M+H]⁺, 499.22. Found: 498.9. HPLC analysis: retention time, 12.4 min; peak area, 95.68%; eluent A, H₂O: eluent B, ACN; gradient elution (100% H₂O to 10% H₂O) over 60 min with flow rate of 0.5 mL/min and detection at 245 nm; column temperature, rt.
Methyl 4-(4-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-
2\textit{H}-pyrazolo[4,3-\textit{d}]pyrimidin-2-yl)butyl)benzoate (386)

Crude acid (300 mg, 0.916 mmol), were used for the next reaction without further characterization where in anhydrous DMF (10 mL) with the acid intermediates were added \textit{N}-methylmorpholine (0.18 mL, 1.65 mmol), and 2-chloro-4,6-dimethoxy-1,3,5-triazine (289.63 mg, 1.65 mmol). The resulting mixtures were stirred at room temperature for 2 h. To this mixture were added \textit{N}-methylmorpholine (0.18 mL, 1.65 mmol), and \textit{L}-glutamate diethyl ester hydrochloride (260.77 mg. 1.28 mmol). The reaction mixtures were stirred for an additional 4 h at room temperature to get 386 in 64% yield (300.6 mg); mp: 219-220.8 °C; \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}6) \delta 10.56 (s, 1H, exch., -NH), 8.66 (d, \textit{J} = 7.5 Hz, 1H, exch., -NH), 7.80 (d, \textit{J} = 8.2 Hz, 2H, Ar), 7.77 (s, 1H, Ar), 7.29 (d, \textit{J} = 8.2 Hz, 2H, Ar), 5.91 (s, 2H, exch., -NH\textsubscript{2}), 4.45-4.38 (m, 1 H, \alpha-CH), 4.23 (t, \textit{J} = 6.9 Hz, 2H, -CH\textsubscript{2}-), 4.13-4.02 (m, 4H, -OCH\textsubscript{2}CH\textsubscript{3}), 2.66 (t, \textit{J} = 7.5 Hz, 2H, -CH\textsubscript{2}-), 2.44 (t, \textit{J} = 7.5 Hz, 2H, \gamma-CH\textsubscript{2}-), 2.13 – 1.96 (m, 2H, \beta-CH\textsubscript{2}-), 1.85-1.79 (p, \textit{J} = 7.5 Hz, 2H, -CH\textsubscript{2}-), 1.57-1.48 (p, \textit{J} = 7.7 Hz, 2H, -CH\textsubscript{2}-), 1.20-1.15 (m, 6H, -OCH\textsubscript{2}CH\textsubscript{3}). Anal. Calcd. for C\textsubscript{25}H\textsubscript{32}N\textsubscript{6}O\textsubscript{6} 0.88 CH\textsubscript{3}OH: C, 57.47; H, 6.62; N, 15.53. Found: C, 57.70; H, 6.41; N, 15.15.

General procedure for synthesis of 297-298 and 301-302.

The compounds 298 and 301-302 from previous steps was added to 1 N NaOH (2 mL), and the hydrolyzed at room temperature for an hour. The mixture was cooled and acidified using 1 N hydrochloric acid. The precipitate was collected and dried under vacuum. Compound 297 and 301 was not sent for biological evaluation and requires further characterization.
(4-(4-(5-amino-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoyl)-L-glutamic acid (298)

Compound 380 (100 mg, 0.19 mmol) was dissolved in MeOH (2 mL) added 1N NaOH (2 mL) and the mixture was stirred under N₂ at room temperature for 10 h. TLC showed the disappearance of the starting material and one major spot at the origin (CHCl₃:MeOH 5:1). The reaction mixture was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3-4 with dropwise addition of 1N HCl. The resulting suspension was frozen in dry ice-acetone bath, thawed to 4-5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P₂O₅ to afford the target compound 298 (41.3 mg, yield 52%) as a white powder, Rf 0.18 (CHCl₃:MeOH: NH₄OH 5:1:0.5); mp, 182 °C;¹H NMR (400 MHz, DMSO-d₆) δ 12.46 (s, 2H, br., exch., -COOH), 8.53 (d, J = 8.2 Hz, 1H, exch., -NH), 7.78 (d, J = 8.2 Hz, 2H, Ar), 7.55 (s, 1H, Ar), 7.27 (d, J = 8.2 Hz, 2H, Ar), 6.62 (s, 2H, exch., -NH₂), 4.46 (t, J = 6.9 Hz, 2H, -CH₂), 4.41-4.36 (m, 1 H, α-CH), 2.64 (t, J = 7.5 Hz, 2H, -CH₂), 2.34 (t, J = 7.5 Hz, 2H, γ-CH₂), 2.12 – 1.90 (m, 2H, β-CH₂), 1.79 (p, J = 7.5 Hz, 2H, -CH₂), 1.49 (p, J = 7.9 Hz, 2H, -CH₂). Anal. Calcd. for C₂₁H₂₄N₆O₆ 0.41 CH₃OH 0.37 HCl: C, 53.23; H, 5.43; N, 17.39. Found: C, 53.20; H, 5.55; N, 17.47.

(4-(4-(5-amino-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoyl)-L-glutamic acid (302)

Compound 386 (150 mg, 0.29 mmol) was dissolved in MeOH (2 mL) added 1N NaOH (2 mL) and the mixture was stirred under N₂ at room temperature for 10 h. TLC showed the disappearance of the starting material and one major spot at the origin (CHCl₃:MeOH 5:1). The reaction mixture was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was
adjusted to 3-4 with dropwise addition of 1N HCl. The resulting suspension was frozen in dry ice-acetone bath, thawed to 4-5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P₂O₅ to afford the target compound 302 (82.8 mg, yield 62%) as a white powder, Rf=0.21 (CHCl₃:MeOH 5:1); mp, 172 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 12.72 (s, 2H, br., exch., -COOH), 10.63 (s, 1H, exch., -NH), 8.54 (d, J = 8.2 Hz, 1H, exch., -NH), 7.81 (d, J = 8.2 Hz, 2H, Ar), 7.76 (s, 1H, Ar), 7.28 (d, J = 8.2 Hz, 2H, Ar), 6.01 (s, 2H, exch., -NH₂), 4.40-4.32 (m, 1 H, α-CH), 4.23 (t, J = 6.9 Hz, 2H, -CH₂-), 2.65 (t, J = 7.5 Hz, 2H, -CH₂-), 2.34 (t, J = 7.5 Hz, 2H, γ-CH₂-), 2.10 – 1.90 (m, 2H, β-CH₂-), 1.86-1.75 (m, 2H, -CH₂-), 1.59-1.46 (m, 2H, -CH₂-). Anal. Calcd. for C₂₁H₂₄N₆O₆ 0.76 HCl: C, 52.08; H, 5.15; N, 17.35. Found: C, 52.07; H, 5.41; N, 17.33.

4-(3-fluoro-4-(methoxycarbonyl)phenoxy)butanoic acid (398)

To 100 mL round bottom flask was added a mixture of compound 399 (3.0 g, 13.45 mmol), cesium carbonate (6.41 g, 19.67 mmol), TBAI (8.23 g, 22.28 mmol) and anhydrous DMF (20 mL). Compound 390 (1.71 g, 10.03 mmol) was added dropwise to the mixture. The reaction mixture was then stirred at room temperature for 2 h at 100 °C. Ethyl acetate was added into the reaction mixture. Combined mixture was washed with two portions of water. After evaporation of solvent under reduced pressure MeOH (20 mL) was added followed by silica gel (1 g). The resulting plug was loaded on to a silica gel column and eluted with 1:10 (ethylacetate:hexane). Fractions with and Rf =0.91 (TLC) (hexane:ethylacetate, 1:1) were pooled and evaporated to afford tert-butyl ester 400 (2.07 g, yield; 66%) as a colorless liquid. ¹H NMR (500 MHz, DMSO-d₆) δ 7.83 (t, J = 8.8 Hz, 1H, Ar), 6.92 (dd, J = 13.2, 2.5 Hz, 1H, Ar), 6.87 (dd, J = 8.8, 2.5 Hz, 1H, Ar), 4.07 (t, J
= 6.4 Hz, 2H, -CH₂-), 3.81 (s, 3H, -OCH₃), 2.36 (t, J = 7.4 Hz, 2H, -CH₂-), 1.94 (p, J = 7.0 Hz, 2H, -CH₂-), 1.40 (s, 9H, -CH₃). Without further characterization trifluoroacetic acid was then added into 400 and mixture was stirred at room temperature in DCM. Excess of trifluoroacetic acid was evaporated and MeOH (20 mL) was added followed by silica gel (1 g). The resulting plug was loaded on to a silica gel column and eluted with 1:10 (ethylacetate:hexane). Fractions with and Rf = 0.48 (TLC) (Hexane:ethylacetate, 1:1) were pooled and evaporated to afford 398 (1.36 g, yield 80%) as white solid, Rf = 0.45 (Hexane:EtOAc, 1:1); mp, 118 C; ¹H NMR (400 MHz, DMSO-d₆) δ 12.19 (s, 1H, exch., COOH), 7.84 (t, J = 8.8 Hz, 1H, Ar), 6.94 (dd, J = 13.2, 2.4 Hz, 1H, Ar), 6.88 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 4.08 (t, J = 6.4 Hz, 2H, -CH₂-), 3.81 (s, 3H, -OCH₃), 2.38 (t, J = 7.3 Hz, 2H, -CH₂-), 1.96 (p, J = 7.0 Hz, 2H, -CH₂-). This compound was used for the next reaction without further characterization.

**Methyl 4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)propoxy)-2-fluorobenzoate (389)**

To 398 (3.20 g, 12.49 mmol) in a 250 mL flask was added oxalyl chloride (9.51 g, 74.93 mmol) and anhydrous CH₂Cl₂ (20 mL). The resulting solution was refluxed for 1 hour and then cooled to room temperature. After the solvent was evaporated under reduced pressure, the residue was dissolved in 20 mL of Et₂O. The resulting solution was added dropwise to an ice cooled diazomethane (generated in situ from 10 g of Diazald® by using Aldrich Mini Diazald® apparatus) in an ice bath over 10 min. The resulting mixture was allowed to stand for 30 min and then stirred for an additional 1 hour. To this solution was added 48% HBr (20 mL). The resulting mixture was refluxed for 1.5 h. After the mixture was cooled to room temperature, the organic layer was separated, and the aqueous layer was extracted with Et₂O (2 X 200 mL). The combined organic
layer and Et₂O extract was washed with two portions of 10% Na₂CO₃ solution and dried over Na₂SO₄. Evaporation of the solvent afforded 403 in 92% yield (2.70 g). To a suspension of 2,6-diaminopyrimidin-4-one 393 (1.11 g, 10 mmol) in anhydrous DMF (25 mL) was added 403 (8.46 mmol). The resulting mixture was stirred under N₂ at room temperature for 3 days. After evaporation of solvent under reduced pressure, MeOH (20 mL) was added followed by silica gel (5 g). The resulting plug was loaded on to a silica gel column and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃. Fractions (CHCl₃:CH₃OH, 5:1) were pooled and evaporated to afford 389 (640 mg, yield 21%) as light orange solid. TLC Rf = 0.40 (CHCl₃:MeOH: NH₄OH, 5:1:0.5); mp, 175-176 °C ¹H NMR (500 MHz, DMSO-d₆) δ 10.91 (s, 1H, exch., 7-NH), 10.19 (s, 1H, exch., 3-NH), 7.86 (t, J = 8.8 Hz, 1H, Ar), 6.95 (dd, J = 13.1, 2.5 Hz, 1H, Ar), 6.89 (dd, J = 8.8, 2.5 Hz, 1H, Ar), 6.02 (s, 2H, exch., -NH₂), 5.91 (s, 1H, C5-CH, Ar), 4.07 (t, J = 6.4 Hz, 2H, -CH₂-), 3.81 (s, 3H, -OCH₃), 2.65 (t, J = 7.4 Hz, 2H, -CH₂-), 2.04 (p, J = 7.0 Hz, 2H, -CH₂-). This compound was used for the next reaction without further characterization.

(4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)propoxy)-2-fluorobenzoyl)-L-glutamic acid (305)

Compound 389 (300 mg, 0.86 mmol) was dissolved in MeOH (10 mL) added 1N NaOH (10 mL) and the mixture was stirred under N₂ at room temperature for 10 h. TLC showed disappearance of the starting material and one major spot at the origin (CHCl₃:MeOH 5:1). The reaction mixture was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3-4 with drop wise addition of 1N HCl. The resulting suspension was frozen in dry ice-acetone bath, thawed to 4-5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P₂O₅ to afford the compound 387 (173 mg,
yield 60%) as yellow solid, Rf 0.20 (5:1 CHCl₃: MeOH). This compound was used for the next reaction without further characterization. To a 250 mL round bottom flask, was added a mixture of compound 387 (150 mg, 0.43 mmol), N-methylmorpholine (0.78 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.79 mmol) and anhydrous DMF (10 mL). The resulting mixture was stirred at room temperature under anhydrous condition for 1.5 h. N-methylmorpholine (0.79 mmol) and L-glutamate di-tert-butyl hydrochloride 388 (123.24 mg, 0.61 mmol) were added in reaction mixture. The resulting mixture was then stirred at room temperature under anhydrous condition for 12 h. After evaporation of solvent under reduced pressure, MeOH (20 mL) was added followed by silica gel (1 g). The resulting plug was loaded on to a silica gel column and eluted with CHCl₃ followed by 1% MeOH in CHCl₃. Fractions with Rf = 0.60 (CHCl₃:CH₃OH, NH₄OH, 5:1:0.5) were pooled and evaporated to afford 404 (142 mg, yield 62%) as a brown sticky solid. Compound 404 (120 mg, 0.22 mmol) was dissolved in MeOH (10 mL) added 1N NaOH (10 mL) and the mixture was stirred under N₂ at room temperature for 10 h. TLC showed the disappearance of the starting material and one major spot at the origin (CHCl₃:MeOH 5:1). The reaction mixture was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3-4 with dropwise addition of 1N HCl. The resulting suspension was frozen in dry ice-acetone bath, thawed to 4-5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P₂O₅ to afford the target compound 305 (58.8 mg, yield 55%) as a white powder, Rf 0.15 (CHCl₃:MeOH:NH₄OH, 5:1:0.5); mp, 148-149.2 °C; ¹H NMR (500 MHz, DMSO-d₆) 𝛿 10.89 (s, 1H, exch., 7-NH), 10.36 (s, 1H, exch., 3-NH), 7.96 (dd, J = 9.3 Hz, 6.1 Hz, 1H, exch., -CONH), 7.78 (t, J = 8.8 Hz, 1H, Ar), 6.91 (dd, J = 13.2, 2.4 Hz, 1H, Ar), 6.87 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 6.10 (s, 1H, exch., -NH₂), 5.90 (s, 1H, C5-CH, Ar), 4.18-4.12 (m, 1H, α-CH), 4.05 (t, J = 6.4 Hz, 2H, -CH₂-), 2.65 (t, J = 7.4 Hz, 2H, -CH₂-), 2.34-
2.13 (m, 2H, γ-CH2-), 2.11-2.02 (m, 2H, β-CH2-), 2.05-1.95 (m, 2H, -CH2-). MS (ESI) m/z calculated for C21H22FN5O7 [M+H]+, 476.15. Found: 475.6. HPLC analysis: retention time, 8.32 min; peak area, 97.57%; eluent A, H2O: eluent B, ACN; gradient elution (100% H2O to 10% H2O) over 60 min with flow rate of 0.5 mL/min and detection at 245 nm; column temperature, rt.

Docking protocol for molecular modeling single agents with combination chemotherapy and multiple RTK inhibitory potential:

Docking of target compounds was carried out in the published X-ray crystal structure of colchicine in tubulin (PDB: 6BS2, 2.65 Å)327, axitinib in VEGFR-2 (PDB: 4AG8, 1.95 Å)329 gefitinib in EGFR (PDB: 4WKQ, 1.85 Å)340, and in the homology model of PDGFR-β294 using Schrodinger Maestro 2020-1.156 The crystal structure of tubulin, VEGFR-2 and EGFR were obtained from the protein database and imported into Schrödinger Maestro. All the compounds were redocked with an RMSD (root-mean-square deviation) of 0.89 and 0.91 for the best-scored pose, thus validating the docking process. All docking procedures were performed using various modules of Schrödinger Maestro suite (Schrödinger, LLC, New York, NY, 2020). The polypeptide structures of in tubulin, EGFR, VEGFR-2 and the homology model of PDGFR-β were optimized and prepared for docking using the Maestro Protein Preparation Wizard to assess bond order and missing hydrogens, followed by energy minimization using the OPLS3e force field. Gaps in the protein structures were not corrected as they were far from the active site. The Maestro induced-fit Grid Generation module was then used to define a 15 × 15 × 15 Å grid from the center of all the ligands. Ligands used in the computational docking study were built using the Maestro 2D Build module. The Maestro LigPrep module was then used to generate conformers of each compound subjected to energy minimization using the OPLS3e force field protocol. The resulting
compounds were docked into the prepared tubulin, EGFR, VEGFR-2 and the homology model of PDGFR-β structures using the Maestro Induced Fit Docking. Induced Fit Docking was performed with standard precision with flexible ligand sampling. A total of 20 initial poses were generated for each compound. Based on the pose score, the top 4 poses were selected and subjected to energy minimization using the OPLS3e force field. Finally, the top 2 poses per compound were generated and ranked according to Glide score, which is an approximation of binding energy defined by receptor-ligand complex energies. The top pose of specific compounds were analyzed and presented in the statement of the problem. Docked scores are listed in Table 33 and Table 34.

**Table 33.** Docked scores of proposed compounds in colchicine site of tubulin, EGFR, VEGFR-2 and PDGFR-β for series I-VII

<table>
<thead>
<tr>
<th></th>
<th>Tubulin (kcal/mol)</th>
<th>EGFR (kcal/mol)</th>
<th>VEGFR-2 (kcal/mol)</th>
<th>PDGFR-β (kcal/mol)</th>
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<tr>
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Table 34. Docked scores of proposed compounds in colchicine site of tubulin for series VIII-XIV

<table>
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<th>Compounds</th>
<th>Docked scores in tubulin (kcal/mol)</th>
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Docking protocol for molecular modeling of selective pjDHFR inhibitors

Docking of target compounds was carried out using the published X-ray crystal structure of N6-methyl-N6-(3,4,5-trifluorophenyl)pyrido[3,2-d]pyrimidine-2,4,6-triamine in hDHFR (PDB: 4QJC, 1.62 Å) and in the homology model of pjDHFR using Schrödinger Maestro suite (Schrödinger, LLC, New York, NY, 2020). The crystal structure of hDHFR (PDB: 4QJC, 1.62 Å) was obtained from the protein database and imported into Schrodinger Maestro. All the compounds were redocked with an RMSD (root-mean-square deviation) of 0.89 and 0.91 for the
best-scored pose, thus validating the docking process. All docking procedures were performed using various modules of Schrödinger Maestro suite. The polypeptide structures hDHFR and the homology model of pjDHFR were optimized and prepared for docking using the Maestro Protein Preparation Wizard to assess bond order and missing hydrogens, followed by energy minimization using the OPLS3e force field. Gaps in the protein structures were not corrected as they were far from the active site. The Maestro induced-fit Grid Generation module was then used to define a $15 \times 15 \times 15$ Å grid from the center of all the ligands. Ligands used in the computational docking study were built using the Maestro 2D Build module. The Maestro LigPrep module was then used to generate conformers of each compound subjected to energy minimization using the OPLS3e force field protocol. The resulting compounds were docked into the prepared hDHFR and the homology model of pjDHFR structures using the Maestro Induced Fit Docking. Induced Fit Docking was performed with standard precision with flexible ligand sampling. A total of 20 initial poses were generated for each compound. Based on the pose score, the top 4 poses were selected and subjected to energy minimization using the OPLS3e force field. Finally, the top 2 poses per compound were generated and ranked according to Glide score, which is an approximation of binding energy defined by receptor-ligand complex energies. The validation of Schrödinger Maestro as a suitable docking system for pjDHFR and hDHFR was carried out by re-docking the native ligands in the x-ray crystal structures of hDHFR (PDB: 4QJC) and homology model of pjDHFR. The best docked pose of the ligands had RMSD of 0.7065 Å in pjDHFR and 0.8802 Å in hDHFR. The top pose of specific compounds were analyzed and presented in the statement of the problem. Docking scores are listed in Table 35.
Table 35. Docked scores of proposed compounds in homology model of pjDHFR and X-ray crystal structure of hDHFR

<table>
<thead>
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<th></th>
<th>hDHFR (kcal/mol)</th>
<th>pjDHFR (kcal/mol)</th>
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<tbody>
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</tr>
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<tr>
<td>PTX</td>
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Docking protocol for molecular modeling of single agents with tumor targeting via cellular uptake by Folate Receptors and/or Proton-Coupled Folate Transporter and inhibition of de novo purine nucleotide biosynthesis:

The X-ray crystal structures of human FRα bound to AGF183 (PDB: 5IZQ, 3.60 Å)\textsuperscript{190}, FRβ bound to PMX (PDB: 4KN2, 2.60 Å)\textsuperscript{388}, human GARFTase bound to AGF150 (PDB: 4ZZ1, 1.35 Å)\textsuperscript{211} and human AICARFTase (PDB: 1P4R, 2.55 Å)\textsuperscript{219} were obtained from the protein database. Docking studies were performed using Schrödinger Maestro suite 2020-1.\textsuperscript{156} The polypeptide structures of FRα, FRβ, GARFTase, AICARFTase and SHMT2 were optimized and prepared for docking using the Maestro Protein Preparation Wizard to assess bond order and missing hydrogens, followed by energy minimization using the OPLS3e force field. Gaps in the protein structures were not corrected as they were far from the active site. The Maestro induced-
fit Grid Generation module was then used to define a $15 \times 15 \times 15$ Å grid from the center of all the ligands. Ligands used in the computational docking study were built using the Maestro 2D Build module. The Maestro LigPrep module was then used to generate conformers of each compound subjected to energy minimization using the OPLS3e force field protocol. The resulting compounds were docked into the prepared FRα, FRβ, GARFTase, AICARFTase and SHMT2 using the Maestro Induced Fit Docking. Induced Fit Docking was performed with standard precision with flexible ligand sampling. A total of 20 initial poses were generated for each compound. Based on the pose score, the top 4 poses were selected and subjected to energy minimization using the OPLS3e force field. Finally, the top 2 poses per compound were generated and ranked according to Glide score, which is an approximation of binding energy defined by receptor-ligand complex energies. The validation of Schrödinger Maestro as a suitable docking system for FRα, FRβ, GARFTase, AICARFTase and SHMT2 was carried out by re-docking the native ligands in the x-ray crystal structures of FRα, FRβ, GARFTase, AICARFTase and SHMT2. The best pose of AGF183 in FRα had an RMSD of 0.96 Å. PMX in FRβ had an RMSD of 0.89 Å, AGF150 in the human GARFTase had an RMSD of 1.15 Å, and ((S)-(4-((2-amino-4-hydroxyquinazoline)-6-sulfonamido)phenyl)(hydroxy)methyl)-L-glutamic acid in the human AICARFTase had an RMSD of 1.10 Å. Thus, Schrödinger Maestro 2020-1 was validated for our docking purposes in FRα, FRβ, GARFTase, AICARFTase and SHMT2. The top pose of specific compounds were analyzed and presented in the statement of the problem. Docking scores are listed in Table 36.
Table 36. Docked scores of proposed compounds in crystal structures of FRα, FRβ, GARFTase and AICARFTase

<table>
<thead>
<tr>
<th></th>
<th>FRα (kcal/mol)</th>
<th>FRβ (kcal/mol)</th>
<th>GARFTase (kcal/mol)</th>
<th>AICARFTase (kcal/mol)</th>
<th>SHMT2 (kcal/mol)</th>
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<tbody>
<tr>
<td>297</td>
<td>-14.98</td>
<td>-15.14</td>
<td>-14.02</td>
<td>-10.55</td>
<td>-10.16</td>
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VI. SUMMARY

This dissertation describes the design and synthesis of single agents with combination chemotherapy and multiple RTK inhibitory potential, selective pjDHFR inhibitors, and single agents with tumor targeting via cellular uptake by Folate Receptors and Proton-Coupled Folate Transporter and inhibition of de novo purine nucleotide biosynthesis. The novel compounds synthesized as part of this study are listed below:

1. \( N\)-(4-methoxyphenyl)-\(N\),2-dimethylthieno[2,3-\(d\)]pyrimidin-4-amine (182)
2. \( N\)-(4-methoxyphenyl)- 2-methylthieno[2,3-\(d\)]pyrimidin-4-amine (183)
3. \( N\),2-dimethyl-\(N\)-(4-(methylthio) phenyl)thieno[2,3-\(d\)]pyrimidin-4-amine (184)
4. 4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)-2-methylthieno[2,3-\(d\)]pyrimidine (185):
5. \( N\)-(5-methoxynaphthalen-2-yl)-\(N\),2-dimethylthieno[2,3-\(d\)]pyrimidin-4-amine (189)
6. \( N\)-(4-methoxyphenyl)-\(N\)-methylthieno[2,3-\(d\)]pyrimidin-4-amine (196)
7. \( N\)4-(4-methoxyphenyl)-\(N\)4-methylthieno[2,3-\(d\)]pyrimidine-2,4-diamine (197)
8. 4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)thieno[2,3-\(d\)]pyrimidine (198)
9. 4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)thieno[2,3-\(d\)]pyrimidin-2-amine (199)
10. 2-chloro-\(N\)-(4-methoxyphenyl)-\(N\)-methylthieno[2,3-\(d\)]pyrimidin-4-amine (204)
11. 2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)thieno[2,3-\(d\)]pyrimidine (205)
12. \( N\)-(4-methoxyphenyl)-\(N\),2,5- trimethylthieno[2,3-\(d\)]pyrimidin-4-amine (218)
13. 2-chloro-\(N\)-(4-methoxyphenyl)-\(N\),5-dimethylthieno[2,3-\(d\)]pyrimidin-4-amine (219)
14. \( N\)4-(4-methoxyphenyl)-\(N\)4,5-dimethylthieno[2,3-\(d\)]pyrimidine-2,4-diamine (220)
15. 4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2,5-dimethylthieno[2,3-\textit{d}]pyrimidine (221)
16. 2-chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-5-methylthieno[2,3-\textit{d}]pyrimidine (222)
17. 4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-5-methylthieno[2,3-\textit{d}]pyrimidin-2-amine (223)
18. \textit{N}-(4-methoxyphenyl)-\textit{N},2,5,6-tetramethylthieno[2,3-\textit{d}]pyrimidin-4-amine (225)
19. \textit{N},2,5,6-tetramethyl-\textit{N}-(4-(methylthio)phenyl)thieno[2,3-\textit{d}]pyrimidin-4-amine (226)
20. \textit{N}-(4-methoxyphenyl)-2,5,6-trimethylthieno[2,3-\textit{d}]pyrimidin-4-amine (227)
21. 2,5,6-trimethyl-\textit{N}-(4-(methylthio)phenyl)thieno[2,3-\textit{d}]pyrimidin-4-amine (228)
22. 7-methoxy-4-(2,5,6-trimethylthieno[2,3-\textit{d}]pyrimidin-4-yl)-3,4-dihydro-2\textit{H}-benzo[\textit{b}][1,4]oxazine (229)
23. Methyl 3-amino-4-benzyl-1\textit{H}-pyrrole-2-carboxylate (323)
24. 4-chloro-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidine (328)
25. 2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidin-4(3\textit{H})-one (329)
26. Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[\textit{b}]thiophene-3-carboxylate (330)
27. 7-benzyl-\textit{N}-(4-methoxyphenyl)-\textit{N}-methyl-5\textit{H}-pyrrolo[3,2-\textit{d}]pyrimidin-4-amine (232)
28. \textit{N}-(4-methoxyphenyl)-\textit{N},2-dimethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidin-4-amine (234)
29. \textit{N},2-dimethyl-\textit{N}-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidin-4-amine (236)
30. 4-(6-methoxy-3,4-dihydroquinolin-1(2\textit{H})-yl)-2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\textit{d}]pyrimidine (238)
31. \(N\)-(5-methoxynaphthalen-2-yl)-\(N\),2-dimethyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-4-amine (239)

32. 2-amino-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-4(3\(H\))-one (336)

33. \(N\)-(4-chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-2-yl)pivalamide (337)

34. \(N\)4-(4-methoxyphenyl)-\(N\)4-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidine-2,4-diamine (244)

35. \(N\)4-methyl-\(N\)4-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidine-2,4-diamine (245)

36. 4-(6-methoxy-3,4-dihydroquinolin-1(2\(H\))-yl)-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-\(d\)]pyrimidin-2-amine (246)

37. \(N\)-(4-methoxyphenyl)-\(N\)-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-4-amine (247)

38. \(N\)-methyl-\(N\)-(4-(methylthio)phenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-\(d\)]pyrimidin-4-amine (248)

39. 5-chloro-\(N\)-(4-methoxyphenyl)-\(N\),1-dimethyl-1\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-amine (251)

40. 5-chloro-\(N\)-(4-methoxyphenyl)-1-methyl-1\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-amine (252)

41. 1-(5-chloro-1-methyl-1\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-yl)-6-methoxy-1,2,3,4-tetrahydroquinoline (253)

42. 5-chloro-\(N\)-(4-ethoxyphenyl)-\(N\),1-dimethyl-1\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-amine (257)

43. 5-chloro-\(N\)-(4-isopropoxyphenyl)-\(N\),1-dimethyl-1\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-amine (258)
44. 5-chloro-N-(5-methoxynaphthalen-2-yl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (261)

45. 5-chloro-N,1-dimethyl-N-(4-(methylthio)phenyl)-1H-pyrazolo[4,3-d]pyrimidin-7-amine (262)

46. 5-chloro-N-(3-fluoro-4-methoxyphenyl)-N,1-dimethyl-1H-pyrazolo[4,3-d]pyrimidin-7-amine (263)

47. 6-(phenylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (282)

48. 6-((3-methoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (283)

49. 6-((4-methoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (284)

50. 6-((3,4-dimethoxyphenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (285)

51. 6-((4-fluorophenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (286)

52. 6-((4-(trifluoromethoxy)phenyl)thio)pyrido[2,3-d]pyrimidine-2,4-diamine (287)

53. 6-(naphthalen-1-ylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (288)

54. 6-(naphthalen-2-ylthio)pyrido[2,3-d]pyrimidine-2,4-diamine (289)

55. Methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-1H-pyrazole-5-carboxylate (372)

56. Methyl 1-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-nitro-1H-pyrazole-3-carboxylate (373)

57. Methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1H-pyrazole-5-carboxylate (370)

58. Methyl 1-(4-(4-(methoxycarbonyl)phenyl)butyl)-4-nitro-1H-pyrazole-3-carboxylate (371)
59. Methyl 4-amino-1-(3-(4-(methoxycarbonyl)phenyl)propyl)-1H-pyrazole-5-carboxylate (375)

60. Methyl 4-amino-1-(3-(4-(methoxycarbonyl)phenyl)propyl)-1H-pyrazole-3-carboxylate (381)

61. Methyl 4-amino-1-(4-(4-(methoxycarbonyl)phenyl)butyl)-1H-pyrazole-5-carboxylate (376)

62. Methyl 4-amino-1-(4-(4-(methoxycarbonyl)phenyl)butyl)-1H-pyrazole-3-carboxylate (382)

63. Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoate (378)

64. Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (383)

65. Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (384)

66. Diethyl (4-(4-(5-amino-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoyl)-L-glutamate (380)

67. Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (385)

68. Methyl 4-(4-(5-((methoxycarbonyl)amino)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)butyl)benzoate (386)

69. (4-(4-(5-amino-7-oxo-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-1-yl)butyl)benzoyl)-L-glutamic acid (298)

70. (4-(3-(5-amino-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)propyl)benzoyl)-L-glutamic acid (301)
71. (4-(4-(5-amino-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d][pyrimidin-2-yl]butyl)benzoyl)-L-glutamic acid (302)

72. 4-(3-fluoro-4-(methoxycarbonyl)phenoxy)butanoic acid (398)

73. Methyl 4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d][pyrimidin-6-yl]propoxy)-2-fluorobenzoate (389)

74. (4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d][pyrimidin-6-yl]propoxy)-2-fluorobenzoyl)-L-glutamic acid (305)

Proposed compounds from the Series I-VII were evaluated for activity against microtubule assembly, EGFR, VEGFR-2 and PDGFR-β. The results revealed an extensive SAR for thieno[2,3-d]pyrimidine. The SAR for 2,4,5, and 6-position of the scaffold is distinct for thieno[2,3-d]pyrimidines and the effects of a substitution on one scaffold cannot be extrapolated to another. Compound 185 was active across all the three angiokinases and was comparable to erlotinib and sunitinib in inhibition of EGFR and VEGFR-2, respectively and 20-fold higher than sunitinib in inhibition of PDGFRβ. It shows an excellent inhibition of all the four targets (microtubule assembly, EGFR, VEGFR-2 and PDGFR-β) and significant improvement in reduction of tumor growth, compared to the positive control-gemcitabine.

Proposed compounds from the Series VIII-XI evaluated for activity against microtubule assembly. The results revealed an extensive SAR for tricyclic thieno[2,3-d]pyrimidine. The SAR for 2- and 4-position of the scaffold is distinct from bicyclic thieno[2,3-d]pyrimidine. Compound 244 was the active most compound in anti-proliferative assay and showed significant improvement in reduction of tumor growth, compared to the positive control-paclitaxel.
Proposed compounds from the Series XII-XIV evaluated for activity against microtubule assembly. The results revealed an extensive SAR for pyrazolo[4,3-$d$]pyrimidine. The SAR for 4-position of the scaffold is impressively significant. Compound 251 was one of the active most compound in inhibition of colchicine binding and inhibition of tubulin assembly and showed significant improvement in reduction of tumor growth, compared to the positive control-paclitaxel.

Proposed compounds from Series XV-XVII were evaluated biologically against pjDHFR and hDHFR enzymes. The evaluation results and hDHFR crystal structures with some of the proposed compounds show the importance of targeting amino acid differences between pjDHFR and hDHFR. Our attempts to obtain a compound similar in selectivity of TMP and higher in potency than TMP led to compounds 283 and 284, 289. Based on the medicinal chemistry approach of carrying out bioisosteric replacement and studying the active site, compounds were obtained with significantly increased potency and/or selectivity.

Biological evaluation of proposed compounds from Series XVIII-XX were carried out in engineered CHO cell lines overexpressing RFC, PCFT, FRα and FRβ. One of the aims for this study was to obtain compounds with selective uptake through FRα and FRβ, compared to RFC. Majority of the proposed compounds showed a selective transport through FRα and FRβ over RFC. Hence, we were able to successfully incorporate selectivity for FRα and FRβ by performing as scaffold-hopping from the pyrrolo[3,2-$d$]pyrimidines. The investigation of the intracellular targets suggests that there is (are) additional intracellular target(s) than GARFTase and/or AICARFTase that the compounds bind to and inhibit.
VII. APPENDIX

A. Biological evaluation of Single agents with combination chemotherapy and multiple RTK inhibitory potential

The biological evaluations reported in this section were performed by Dr. Michael Ihnat (Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, Oklahoma City, OK 73117), Dr. Ernest Hamel (Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, Frederick National Laboratory for Cancer Research, National Cancer Institute, Frederick, MD 21702), Dr. Susan Mooberry (Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229) and National Cancer Institute (Developmental Therapeutics Program).

A1. Biological evaluation of 2, 4-substituted thieno[2,3-d]pyrimidines

(a) Activity as MTAs

The EC$_{50}$ (concentration required to cause 50% loss of cellular microtubules) was determined in A-10 cells.$^{112}$ The effects of the compounds on interphase and mitotic microtubules were evaluated using indirect immunofluorescence techniques, and the EC$_{50}$ values were calculated from a minimum of three experiments.$^{99}$ Antiproliferative effects were evaluated against the drug sensitive MDA-MB-435 melanoma cells using sulforhodamine B assay and the IC$_{50}$ values (concentration required to cause 50% inhibition of proliferation) were calculated.$^{99}$
Table 37. Effects in cellular assays and on purified tubulin for Series I-IV (ND= not determined)

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>IC₅₀ ± SD in MDA-435 Cells (nM)</th>
<th>EC₅₀ for Microtubule Depolymerization in A-10 Cells (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>183</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>ND</td>
<td>&gt;10 µM</td>
</tr>
<tr>
<td>182</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>8.6 ± 1.1</td>
<td>15.4</td>
</tr>
<tr>
<td>196</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>13.1 ± 1.4</td>
<td>80</td>
</tr>
<tr>
<td>197</td>
<td>NH₂</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>11.0 ± 1.2</td>
<td>46.8</td>
</tr>
<tr>
<td>204</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>6.3 ± 0.5</td>
<td>23</td>
</tr>
<tr>
<td>218</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>2.1 ± 0.2</td>
<td>2.5</td>
</tr>
<tr>
<td>219</td>
<td>Cl</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>2.0 ± 0.3</td>
<td>2.3</td>
</tr>
<tr>
<td>220</td>
<td>NH₂</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>4.3 ± 0.3</td>
<td>7.5</td>
</tr>
<tr>
<td>225</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>10.7 ± 1.0</td>
<td>18</td>
</tr>
<tr>
<td>227</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>ND</td>
<td>&gt;10 µM</td>
</tr>
<tr>
<td>184</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>4.0 ± 0.5</td>
<td>10</td>
</tr>
<tr>
<td>226</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>34.8 ± 3.7</td>
<td>128</td>
</tr>
<tr>
<td>228</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>ND</td>
<td>&gt;10 µM</td>
</tr>
<tr>
<td>185</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>4.2 ± 0.3</td>
<td>8.0</td>
</tr>
<tr>
<td>198</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>14.3 ± 1.9</td>
<td>40</td>
</tr>
<tr>
<td>199</td>
<td>NH₂</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>9.5 ± 0.5</td>
<td>15.2</td>
</tr>
<tr>
<td>205</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>1.5 ± 0.3</td>
<td>2.41</td>
</tr>
<tr>
<td>221</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>-</td>
<td>9.2 ± 1.3</td>
<td>12.2</td>
</tr>
<tr>
<td>222</td>
<td>Cl</td>
<td>CH₃</td>
<td>H</td>
<td>-</td>
<td>16.8 ± 2.2</td>
<td>19.9</td>
</tr>
<tr>
<td>223</td>
<td>NH₂</td>
<td>CH₃</td>
<td>H</td>
<td>-</td>
<td>7.4 ± 1.0</td>
<td>12.9</td>
</tr>
<tr>
<td>229</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>-</td>
<td>202 ± 23</td>
<td>1.2 µM</td>
</tr>
<tr>
<td>189</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>3.9 ± 0.2</td>
<td>5.5</td>
</tr>
<tr>
<td>CA-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.4 ± 0.6</td>
<td>9.8</td>
</tr>
</tbody>
</table>
Biological evaluation of 182-189, 196-199, 204-205, 218-223 and 225-229 enabled a SAR on the thieno[2,3-d]pyrimidine scaffold. An electron withdrawing substituent at the 2-position showed an improvement in inhibition of tubulin depolymerization (Table 37). The activity trend for 2-postion was Cl>CH<sub>3</sub>><NH<sub>2</sub>> H. Varying aryl group at the 4-position on the thieno[2,3-d]pyrimidine scaffold did not create a significant impact on the inhibition of tubulin assembly. Next, the effects of methyl group at 5-positon was evaluated. The most striking effect was seen on comparison of 5-H (182, 204, 197) and 5-CH<sub>3</sub> (218, 219, 220) compounds. Comparing the IC<sub>50</sub> of 182 (2-CH<sub>3</sub>, 5-H) vs. 218 (2-CH<sub>3</sub>, 5-CH<sub>3</sub>); 204 (2-Cl, 5-H) vs. 219 (2-Cl, 5-CH<sub>3</sub>) and 197 (2-NH<sub>2</sub>, 5-H) vs. 220 (2-NH<sub>2</sub>, 5-CH<sub>3</sub>), the 5-CH<sub>3</sub> substitution exhibits an increase in IC<sub>50</sub> values from 3- to 4-fold.

![Figure 111](image1.png)

**Figure 111.** Possible steric clash between N<sup>4</sup>-CH<sub>3</sub> and 5-H in 182; and N<sup>4</sup>-CH<sub>3</sub> and 5-CH<sub>3</sub> in 218.

The improvement in the microtubule depolymerization activity of the 5-CH<sub>3</sub> compounds (218, 219, 220) compared to 5-H compounds (182, 204, 197) can be explained by studying the stable solution conformations of 218 and 183, using <sup>1</sup>HNMR. This solution conformation of 182 is possibly due to steric clash between 5-H and N<sup>4</sup>-CH<sub>3</sub>. This clash is further reinforced in 218 by
steric clash between 5-CH$_3$ and $N^4$-CH$_3$ (Figure 111). On the other hand, 5-CH$_3$ compounds (221, 222, 223) compared to 5-H compounds (185, 205, 199) had almost 2-fold less potency. The 5,6-dimethyl substituted compounds (225-229) compared to desmethyl compounds (182-185) were not conducive to microtubule depolymerization activity. The biological evaluation of 225 (Table 37) had comparable potency to 182, and on the other hand, compound 226 causes 8-fold decrease in antiproliferative activity against MDA-MB-435 cells and 12-fold decrease in inhibition of microtubule depolymerization. The SAR study from methyl group incorporation thus suggested that, methyl groups at only 5-position improves potency on the contrary methyl groups at both the 5- and 6-position are detrimental for anti-tubulin activity.

**Figure 112.** *in vivo* study of 225 in mice: (A) Tumor volumes during the trial on treatment with 225 (10 mg/kg), Paclitaxel (PTX) and control, dosed i.p. Arrows indicate treatment days for compound 7 and PTX (dosed days 1, 3, 5, 8, 10, 12). n = 7-10 tumors and data points represent mean ± SEM. (B) Change in weight of the animal on treatment with 225 (10 mg/kg), Paclitaxel (PTX) and control.
One of the most active compound from the thieno[2,3-\(d\)]pyrimidine series (225) was evaluated \textit{in vivo} using MDA-MB-435 model (Figure 112). The MDA-MB-435 cells were implanted subcutaneously into both flanks of nude mice. Mice were treated with MTD and schedule using IP injections. Tumor volume was determined using tumor length, width, and height measured with calipers. Two-way repeated measures ANOVA with post hoc testing was used to analyze the datasets. Compared to paclitaxel, 225 showed a significant improvement in reduction of tumor growth. It also showed a weight loss of only 5%. Further evaluation for this compound in other preclinical models is underway.

\textbf{(b) Activity as angiokinese inhibitors}

Cells used were tumor cell lines naturally expressing high levels of VEGFR-2 (U251), PDGFR-b (SF-539) and EGFR (A431).\textsuperscript{99} Expression levels at the RNA level were derived from the NCI Developmental Therapeutics Program (NCI-DTP) web site describing molecular target information. Briefly, cells at 60–75\% confluence were placed in serum-free medium for 18 h to reduce background phosphorylation. Cells were always >98\% viable by trypan blue exclusion. Cells were then pretreated for 60 min to obtain dose–response data, using concentrations of 1.4–100 \(\mu\)M compound, followed in \(\frac{1}{3}\log\) increments by 100 ng/mL VEGF, PDGF-BB or EGF for 10 min. The reaction was stopped, and cells permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05\% Triton X-100, protease inhibitor cocktail and tyrosine phosphatase inhibitor cocktail. The TBS solution was then removed and cells fixed to the plate for 30 min at 60 \(^\circ\)C with a further incubation in 70\% ethanol for 30 min. Cells were exposed to a blocking solution (TBS with 1\% BSA) for 1 h, washed, and then a horseradish peroxidase (HRP)-conjugated phosphotyrosine (PY) antibody was added overnight.
The antibody was removed, and the cells were washed again in TBS, exposed to an enhanced luminol ELISA substrate (Pierce Chemical EMD, Rockford, IL), and light emission was measured using a UV Products (Upland, CA) BioChemi digital darkroom. Data were graphed as a percent of cells receiving growth factor alone and IC\textsubscript{50} values were determined from two to three separate experiments (n = 8–24) using non-linear regression dose–response analysis with Prism 5.0 software (GraphPad, San Diego, CA). In each case, the activity of a positive control inhibitor did not deviate more than 10% from the IC\textsubscript{50} values listed in the table.

Table 38. Inhibition (IC\textsubscript{50}) of cellular VEGFR-2, EGFR and PDGFRβ- RTKs. (ND= not determined)

<table>
<thead>
<tr>
<th></th>
<th>EGFR (nM)</th>
<th>VEGFR-2 (nM)</th>
<th>PDGFRβ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>182</td>
<td>18.9±1.5</td>
<td>59.1±5.4</td>
<td>41.4±10.8</td>
</tr>
<tr>
<td>184</td>
<td>64.9±10.3</td>
<td>436.2±56.1</td>
<td>182.1±26.2</td>
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<tr>
<td>185</td>
<td>12.6 ± 1.2</td>
<td>25.2 ± 7.2</td>
<td>35.2 ± 5.8</td>
</tr>
<tr>
<td>189</td>
<td>28.8±4.1</td>
<td>52.2±5.4</td>
<td>25.6±4.6</td>
</tr>
<tr>
<td>196</td>
<td>50.2±6.8</td>
<td>188.4±26.9</td>
<td>185.6±32.0</td>
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<tr>
<td>199</td>
<td>30.3±5.1</td>
<td>79.4±2</td>
<td>186±22.2</td>
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<tr>
<td>205</td>
<td>30.9±5.1</td>
<td>41.8±21</td>
<td>23.4±2.6</td>
</tr>
<tr>
<td>225</td>
<td>81.2±10.2</td>
<td>416.5±38.0</td>
<td>140.1±18.9</td>
</tr>
<tr>
<td>226</td>
<td>111.0±17.5</td>
<td>1636.9±206.2</td>
<td>490.3±66.3</td>
</tr>
<tr>
<td>sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
</tr>
<tr>
<td>erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7±18.2</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

The most active anti-tubulin compounds (Table 37) were evaluated for activity against VEGFR-2 (U251), PDGFR-b (SF-539) and EGFR (A431) using cellular assays (Table 38). Since only a few compounds were evaluated, a comprehensive SAR is not possible. The 2-CH\textsubscript{3} of 182
displayed an increased the potency towards all the three kinases compared to 2-H compound (196). Comparison of 182 vs. 196 suggests that methylation at the 5-position of the thieno[2,3-\(d\)]pyrimidine scaffold increases potency for all the EGFR (by 3-fold), VEGFR-2 (by 3-fold) and PDGFR-\(\beta\) (by 5-fold). It also corroborates with the results of molecular modeling studies carried out for these compounds in the colchicine site of tubulin and ATP-binding sites of EGFR, VEGFR-2 and PDGFR-\(\beta\).

The trend of activity in inhibition of microtubule assembly and kinases at the 2-position was: \(\text{CH}_3\text{>Cl}>\text{NH}_2\). This could be due to an increase in cLogP (measure of lipophilicity) for \(\text{CH}_3\text{>Cl}>\text{NH}_2\), which enables a higher passive diffusion of compounds into cancer cells. Compound 185 was active across all the three angiokinases and showed an excellent inhibition of all the four targets (microtubule assembly, EGFR, VEGFR-s and PDGFR-\(\beta\)). Compound 185 was comparable to erlotinib in inhibition of EGFR and 5-fold more potent VEGFR-2. Compound 185 was 14-and 2.5-fold more potent than sunitinib, in inhibition of EGFR and PDGFR\(\beta\), respectively.
**Figure 113:** Effects of 185 versus gemcitabine on primary tumor growth and animal weight in the BxPC-3 flank xenograft model. (A) BxPC-3 tumor bearing NCr nu/nu athymic mice were treated IP with solvent (10% Kolliphor, 10% DMSO in normal saline) or with 185 (8 mg/kg, twice weekly) or with gemcitabine (75 mg/kg, twice weekly) and primary tumor volumes assessed. Statistical analysis was performed with two-way ANOVA and Dunnett’s multiple comparisons test. (B) Animal weights were graphed as percent weight change at day 35 versus the starting weight. Statistical analysis was performed with one-way ANOVA with Tukey’s multiple comparisons test. n = 8 (4 male; 4 female); ns = not significant, *P<0.05, ** P<0.01, ***P<0.001 ****P<0.0001 versus solvent (or gemcitabine, if designated) group.

One of the best compounds 185 which showed activity in all four cell lines of tubulin, EGFR, VEGFR-2 and PDGFR-β was further carried out to *in vivo* animal study (Figure 113). Compound 185 inhibited tumor growth significantly compared to control- gemcitabine.
A2. Biological evaluation of 2- and 4-substituted 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine activity as MTAs

Table 39. Effects in cellular assays and on purified tubulin for Series VIII and XI

<table>
<thead>
<tr>
<th>R1</th>
<th>IC$_{50}$ ± SD in MDA-435 Cells (nM)</th>
<th>EC$_{50}$ for Microtubule Depolymerization in A·10 Cells (nM)</th>
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<tbody>
<tr>
<td>234</td>
<td>CH$_3$</td>
<td>53.0 ± 0.3</td>
</tr>
<tr>
<td>244</td>
<td>NH$_2$</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td>247</td>
<td>H</td>
<td>36.8 ± 5.2</td>
</tr>
<tr>
<td>236</td>
<td>CH$_3$</td>
<td>87.7 ± 4.7</td>
</tr>
<tr>
<td>245</td>
<td>NH$_2$</td>
<td>38.6 ± 5.6</td>
</tr>
<tr>
<td>248</td>
<td>H</td>
<td>ND</td>
</tr>
<tr>
<td>238</td>
<td>CH$_3$</td>
<td>125 ± 14</td>
</tr>
<tr>
<td>246</td>
<td>NH$_2$</td>
<td>59.6 ± 11.8</td>
</tr>
<tr>
<td>239</td>
<td>CH$_3$</td>
<td>81.3 ± 8.4</td>
</tr>
<tr>
<td>240</td>
<td>CH$_3$</td>
<td>ND</td>
</tr>
<tr>
<td>CA-4</td>
<td></td>
<td>3.4 ± 0.6</td>
</tr>
</tbody>
</table>

Biological evaluation of 234, 236, 238, 239, 240, 244-248 enabled a SAR around 2- and 4-position of tricyclic thieno[2,3-d]pyrimidine scaffold. The trend observed at the 2-position was: NH$_2$>H>CH$_3$ for 234, 244, 247 and NH$_2$>CH$_3$ for rest of the compounds (Table 39). This suggests that electron donating group at 2-position is conducive to the activity towards tubulin. In the molecular modeling studied the electron donating group at 2-position is oriented towards the solvent and polar residues. It could also indicates increase in inhibition of tubulin depolymerization. Compared to bicyclic thieno[2,3-d]pyrimidine scaffold, the 2-NH$_2$ showed an
improved activity as MTA. This suggests that similar substitutions on different scaffolds can create varying effects on the targets. It is not therefore advisable to postulate the effect of a substituent form one scaffold to another. Varying the aryl group at the 4-position on the tricyclic thieno[2,3-

\[
d]pyrimidine scaffold did not create a significant impact on the inhibition of tubulin assembly.

**Figure 114.** *in vivo* study of **244** in mice: (a) Tumor volumes during the trial on treatment with **244** (50 mg/kg), Paclitaxel (PTX) and control, dosed i.p. Arrows indicate treatment days for compound 2 and PTX (dosed days 1, 3, 5, 8, 10, 12). n = 7-10 tumors and data points represent mean ± SEM. (b) Change in weight of the animal on treatment with **244** (50 mg/kg), Paclitaxel (PTX) and control.

Based on the solubility studies of the most active compounds from the thieno[2,3-

\[
d]pyrimidine series, **244** was selected for evaluation *in vivo* using MDA-MB-435 model (Figure 114). The MDA-MB-435 cells were implanted subcutaneously into both flanks of nude mice. Mice were treated with MTD and schedule using IP injections. Tumor volume was determined using tumor length, width, and height measured with calipers. Two-way repeated measures ANOVA with post hoc testing was used to analyze the datasets. Compared to paclitaxel, **244** showed a
significant improvement in reduction of tumor growth. It also showed a weight loss of less than 5%. Further evaluation for his compound in other preclinical models is underway.

A3.1 Biological evaluation of 2, 4- and 5-substituted pyrazolo[4,3-\textit{d}]pyrimidines

**Table 40.** Effects in cellular assays and on purified tubulin for Series XII-XIV

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>Inhibition of colchicine binding</th>
<th>Inhibition of tubulin assembly IC₅₀ (μM ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 μM inhibitor</td>
<td>% inhibition ± SD</td>
<td></td>
</tr>
<tr>
<td>0.5 μM inhibitor</td>
<td>% inhibition ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>251</td>
<td>CH₃</td>
<td>CH₃</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>252</td>
<td>H</td>
<td>CH₃</td>
<td>7.1 ± 3</td>
</tr>
<tr>
<td>257</td>
<td>-</td>
<td>CH₂CH₃</td>
<td>99 ± 0.4</td>
</tr>
<tr>
<td>258</td>
<td>-</td>
<td>CH(CH₃)₂</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>253</td>
<td>-</td>
<td>-</td>
<td>94 ± 0.4</td>
</tr>
<tr>
<td>261</td>
<td>-</td>
<td>-</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>262</td>
<td>-</td>
<td>-</td>
<td>93 ± 0.5</td>
</tr>
<tr>
<td>263</td>
<td>-</td>
<td>-</td>
<td>ND*</td>
</tr>
<tr>
<td>CA-4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND*: Not determined

Biological evaluation of 251-253, 257-258, and 261-263 enabled a SAR around 4- position of pyrazolo[4,3-\textit{d}]pyrimidine scaffold (Table 40). Except for 252, all the compounds at 5 μM inhibited [³H]colchicine binding to the protein, and the extent of inhibition was similar to that
obtained with CA-4. With equal or more than 94% and 68% inhibition at 5 and 0.5 μM, respectively, compounds 251, 253, 257 and 263 showed similar potency to the lead compounds and CA-4 (Table 36). Homologation with a single methylene at 4’-position of 151 (4’-OMe) afforded compound 257 (4’-OEt) which was more potent (99% at 5 μM and 88% at 0.5 μM) than compound 251 and CA-4 in the colchicine binding inhibition assay. On the other hand, branching with isopropyl moiety yielded compound 258 which was slightly less potent than 252, with 85% inhibition occurring at 5 μM. Introduction of fluorine atom at 3’-position of the phenyl ring of 251 yielded compound 263, which had 97 and 77% inhibition at 5 and 0.5 μM, respectively, in colchicine binding assay. In the colchicine inhibition assay, compounds 253, 261, 257-258, 262-263 at 5 μM, inhibited the binding of [3H]colchicine by 85–99%. Compound 251 was completely inactive in inhibiting the colchicine binding. Compound 251 lacking the N7-methyl moiety did not inhibit tubulin assembly, emphasizing the essential role of this methyl moiety at N7-position in retaining the bioactive conformation for the anti-tubulin activity.

Figure 115: In vivo data of 251 in mice

One of the best compound from pyrazolo[4,3-d]pyrimidine was evaluated for in vivo activities in mice (Figure 115). Treatment with compound 251 decreased primary tumor growth in
MCF-7 TUBB3 (beta III tubulin overexpressing) breast cancer cell model. * = P<0.05; *** P<0.001 (0.0007 for control vs 251, 0.0003 for PTX vs 251). A) 1x01exp7 cells/100 uL Matrigel implanted into fat pad #4 of 8 weeks old athymic female mice were treated with compound 251 with MTD of 30 mg/kg two times weekly, or paclitaxel at its MTD of 10 mg/kg/week and tumor volumes were determined. Statistical analysis was performed with two-way ANOVA repeated measures post test. Compound 251 is significantly (P<0.0001) better than paclitaxel at reducing MCF-7 TUBB3 (beta III tubulin overexpressing). B) Animal weights were graphed as percent weight change at day 41 over the starting weight. Statistical analysis was performed with one-way ANOVA.

Only the control and paclitaxel mice lost weight at the end. Sample sizes are 7 for control (lost an animal in each group toward the end) and 8 for PTX and 251. Compound 251 was selected for an in vivo xenograft mouse study considering its nanomolar potency in vitro in the NCI cancer cell line panel and its potent activities in inhibiting microtubule polymerization and colchicine binding assay.

A3.2 Evaluation as pan assay interference compounds (PAINS) of 2, 4- and 5-substituted pyrazolo[4,3-d]pyrimidines

PAINs produce often unknowingly false positives that could be a challenge in drug discovery research. As a result, we have carried out an analysis of PAINS and the compounds library was submitted to https://www.cbligand.org/PAINS to filter out structures (molecular weight 290-354 Da). All fragments were classified as "Accepted" due to no structural alerts and passing through the filter. All the tested compounds showed zero PAINS alert and can be used as drug compounds in the future. Theses chemical compounds will give less false positive results in
There will be more specific binding with one desired target rather than nonspecific binding with numerous biological targets. Their identification will be helpful in implementing the pyrazole sub structure as an important step toward the identification of future drug candidates.

**Figure 116**: Pan Assay Interference Compounds (PAINS) results of compounds 251-253, 257-258 and 261-263.

**B. Biological evaluation of Selective pjDHFR inhibitors**

All the proposed compounds were evaluated as inhibitors of recombinant pjDHFR and recombinant hDHFR. Selectivity ratios were determined using recombinant hDHFR as a mammalian DHFR. TMP and PTX were used as positive controls in the assays.

Assays to assess inhibitory concentrations (IC\textsubscript{50}, in nM) against recombinant DHFR from *P. jirovecii* (pj) and human (h) were carried out at 37 °C under 9 µM dihydrofolic acid concentration. The assay also contains 117 µM NADPH, 8.9 mM 2mercaptoethanol, 150 mM
KCl, 41 mM Na phosphate buffer pH 7.4 and sufficient enzyme to cause a change in OD340 of 0.005/minute. The standard error of the mean for these values is 12% or less than the mean value.

The biological evaluations were performed by Dr. Sherry Queener (Indiana University School of Medicine, Indianapolis, IN 46202), Dr. Vivian Cody (Department of Structural Biology, Structural Biology Department, School of Medicine and Biomedical Science, Buffalo, NY 14203), Dr. Melanie Cushion (Infectious Diseases, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267 and The Cincinnati Veterans Medical Center, Cincinnati, OH) and Dr. David Seybert (Chemistry and Biochemistry, Bayer School of Natural and Environmental Sciences, Duquesne University, Pittsburgh PA 15282).

**B1. Biological evaluation of 6-(arylthio)pyrido[2,3-d]pyrimidine-2,4-diamines as selective pjDHFR inhibitors**

Table 41 displays the IC$_{50}$ of compounds 265, 267-269 and 282-289 for pjDHFR and hDHFR. The 6-(arylthio)pyrido[3,2-d]pyrimidines 282 and 284 showed a 88- and 250-fold improvement in selectivity than the 6- amine linked compounds 265 and 267, respectively. The sulfur linked analogs showed a single digit nanomolar and picomolar potency for pjDHFR. Replacement of -NH- of 269 with -S- in 289 improved potency for pjDHFR by 800-fold selectivity ratio for pjDHFR by 28-fold. TMP is widely used because of its exceptional selectivity ratio for pjDHFR but has a low potency for pjDHFR. Compounds with picomolar potency for pjDHFR have not been reported in the literature, in our opinion. All the compounds tested in this series so far have a single digit nM or a pM IC$_{50}$ for pjDHFR.
Table 41. Inhibition Concentrations (IC₅₀) against pjDHFR and hDHFR and Selectivity Ratios for Series XV-XVII

<table>
<thead>
<tr>
<th>#</th>
<th>R</th>
<th>pjDHFR (nM)</th>
<th>hDHFR (nM)</th>
<th>Selectivity [hDHFR/pjDHFR]</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>265</td>
<td>H</td>
<td>300</td>
<td>190</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>267</td>
<td>4’-OCH₃</td>
<td>400</td>
<td>3650</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>268</td>
<td>2’,3’-(CH)₄</td>
<td>250</td>
<td>2100</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>269</td>
<td>3’,4’-(CH)₄</td>
<td>400</td>
<td>2200</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>282</td>
<td>H</td>
<td>29</td>
<td>475</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>283</td>
<td>3’-OCH₃</td>
<td>12.2</td>
<td>335</td>
<td>4090</td>
<td></td>
</tr>
<tr>
<td>284</td>
<td>4’-OCH₃</td>
<td>7.5</td>
<td>17.4 µM</td>
<td>2320</td>
<td></td>
</tr>
<tr>
<td>285</td>
<td>3’,4’-OCH₃</td>
<td>2.0</td>
<td>226</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>286</td>
<td>4’-F</td>
<td>7.5</td>
<td>817</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>287</td>
<td>4’-OCF₃</td>
<td>30</td>
<td>2490</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>288</td>
<td>2’,3’-(CH)₄</td>
<td>2.5</td>
<td>27</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>289</td>
<td>3’,4’-(CH)₄</td>
<td>0.5</td>
<td>70</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>TMP</td>
<td></td>
<td>92</td>
<td>24500</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td>PTX</td>
<td></td>
<td>41</td>
<td>2</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>
Figure 83: A. Docked pose of 289 (green) in the crystal structure of hDHFR (PDB: 4QJC, 1.62 Å). 83B. Docked pose of 289 (green) in the homology model of pjDHFR.

Table 42. Bond angles and bond distance for C-X-C angle and C-X bond measured with Schrödinger Maestro.156

<table>
<thead>
<tr>
<th>Entry</th>
<th>X</th>
<th>C-X Bond distance (Å)</th>
<th>C-X-C Bond angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>269</td>
<td>NH</td>
<td>1.46</td>
<td>120</td>
</tr>
<tr>
<td>289</td>
<td>S</td>
<td>1.78</td>
<td>94.1</td>
</tr>
</tbody>
</table>

This gain in selectivity ratio in -S-linked compounds 282-289 could be explained by studying the active sites of hDHFR and pjDHFR. The binding site where the side chain aryl group binds contains the Met33 in pjDHFR and the Phe31 in hDHFR. The Met33/Phe31 in pjDHFR/hDHFR can affect binding due to their distinct steric and electronic effects. Compared to
the lead compound 269, sulfur linked analog 289 could change the bond angle, distance (Table 42) and electronics of the naphthyl group and cause a clash with Phe31 in hDHFR, whereas appropriately fit with Met33 in pjDHFR (Figure 83). The increased C-S bond length and reduced C-S-C bond angle poses an increase in probability of a clash between the side chain aryl ring and the Phe31 in hDHFR, and thus decrease binding of such compounds with hDHFR. Such a clash is avoided in binding of 6-substituted sulfur linked pyrido[2,3-\(d\)]pyrimidines to pjDHFR active site due to the flexible nature of the Met33 side chain.

**DHFR inhibitory assays**\(^{134}\) (performed by David W. Seybert):

For analysis of inhibitor susceptibility, DHFR was assayed using a continuous spectrophotometric assay. The assay contains a final concentration of 9 \(\mu\)M dihydrofolic acid, 117 \(\mu\)M NADPH, and 6–8 IU of enzyme activity (1 IU D0.005 optical density units/min). The reaction is incubated at 37 °C and change in absorbance at 340 nM, which is related to oxidation of NADPH, is measured over 3 min. The assay under standard conditions is linear over this time span and linear with protein concentration. KCl 150 mM is included in assays of mammalian DHFR. The standard error of the means for these values are 12% or less than the mean value.

**C. Folate receptors (FRs) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one carbon (1C) metabolism inhibitors**

**C1. N1-substituted 5-amino-1,6-dihydro-7\(H\)-pyrazolo[4,3-\(d\)]pyrimidin-7-ones**

The pyrazolo[4,3-\(d\)]pyrimidine compounds 297-298 were designed based on 5-substituted pyrrolo[3,2-\(d\)]pyrimidines 294-295 (Table 43). The synthesized analog 298 showed a considerable potency in FR\(\alpha\) and FR\(\beta\) assays. One of the aims for this study was to obtain compounds with
selective uptake through FRα and FRβ, compared to RFC. Compounds 298 shows a favorable uptake though both the FRs. For 298, no RFC uptake is observed, suggesting that the selectivity of transport through FRα and FRβ. Hence, we have been able to incorporate selectivity for FRα and FRβ by performing as scaffold-hopping from the pyrrolo[3,2-d]pyrimidines 295 to pyrazo[4,3-d]pyrimidine 298. This compound displays can be served as a future lead candidate in designing compounds with the novel scaffold with pyrazolo[4,3-d]pyrimidine.

**Table 43.** IC₅₀ values (in nM) in RFC-, PCFT- and FR-expressing cell lines and KB human tumor cells (express RFC, FRα, and PCFT) for Series XVIII

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFRα</th>
<th>hFRβ</th>
<th>hPCFT</th>
<th>hRFC/ hFRα/hPCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>R2</td>
<td>RT16</td>
<td>D4</td>
<td>R2/hPCFT4</td>
</tr>
<tr>
<td>294</td>
<td>43</td>
<td>&gt;1000</td>
<td>50</td>
<td>1.59</td>
<td>25.2</td>
</tr>
<tr>
<td>295</td>
<td>516</td>
<td>&gt;1000</td>
<td>2.13</td>
<td>1.29</td>
<td>309</td>
</tr>
<tr>
<td>297</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>298</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>6.46</td>
<td>5.91</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60(8)</td>
<td>13.2(2.4)</td>
</tr>
</tbody>
</table>

PMX 894(93) 138(13) 42(9) 60(8) 13.2(2.4) 68 (12)
Figure 117. Cell proliferation assays in MIA-PaCa-2 cells to identify intracellular targets of 298.

The intracellular targets for 294 and 295 were GARFTase and AICARFTase. To investigate the intracellular targets of 298, the compound was subjected to growth inhibition studies of KB cells along with protection by excess folic acid, AICA, adenine and thymidine. To identify the targeted pathways and the folate-dependent intracellular enzymes in KB cells treated with 298 (1–1000 nM), cell proliferation assays were performed in the presence of 10 μM thymidine, 60 μM adenosine, or 320 μM AICA. The results were normalized to those for untreated cells (no drug) and for 298. The results shown are representative of triplicate experiments (Figure 117). From the protection studies performed, the inhibition of proliferation of KB cells was not protected by thymidine suggesting it is not a TS inhibitor. The inhibition of proliferation of KB cells was not protected by AICA alone; suggesting it is might be GARFTase inhibitor, along with another intracellular target. The inhibition of proliferation of KB cells was protected by adenosine at low concentrations (up to 10 nM), suggesting it could be GARFTase and/or AICARFTase inhibitor at that low concentration. At higher concentrations (> 31.6 nM), the inhibition of proliferation of KB cells was not protected by adenosine, suggesting that there is (are) additional
intracellular target(s) that the compounds bind and inhibit. Further studies to investigate the possible targets are underway.

C2. N2-substituted 5-amino-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-ones

Table 44. IC_{50}s (in nM) in RFC-, PCFT- and FR-expressing cell lines and KB human tumor cells (express RFC, FRα, and PCFT) for Series XIX.

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFRα</th>
<th>hFRβ</th>
<th>hPCFT</th>
<th>hRFC/FRα/hPCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>R2</td>
<td>RT16</td>
<td>D4</td>
<td>R2/hPCFT4</td>
</tr>
<tr>
<td>299</td>
<td>649(38)</td>
<td>6.3(1.6)</td>
<td>5.6(1.2)</td>
<td>23.0(3.3)</td>
<td>141 ± 40</td>
</tr>
<tr>
<td>300</td>
<td>&gt;1000</td>
<td>6.3(1.6)</td>
<td>10(2)</td>
<td>213(28)</td>
<td>57.4 ± 21.4</td>
</tr>
<tr>
<td>301</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>302</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>1.63</td>
<td>7.83</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60(8)</td>
<td>13.2(2.4)</td>
</tr>
</tbody>
</table>

The pyrazolo[4,3-d]pyrimidine compounds 301-302 with N2-side chain substitutions were designed by scaffold hopping from 6-substituted pyrrolo[2,3-d]pyrimidine counterparts 299-300. Compared to their pyrrolo[2,3-d]pyrimidine analog 300, the synthesized analog 302 improve binding to 6- and 27-fold in FRα and Frβ, respectively (Table 44). They also have a decreased uptake through RFC and PCFT, compared to the lead compounds 300. Except for loss in PCFT activity of 302, this compound display the desired improvement in antiproliferative activity and transporter selectivity through Frα and Frβ. To study the intracellular targets, detailed protection studies of all the synthesized compounds are currently underway.
C3. Evaluation as pan assay interference compounds (PAINS) of \( N_1 \)-substituted 5-amino-1,6-dihydro-7\( H \)-pyrazolo[4,3-\( d \)]pyrimidin-7-ones and \( N_2 \)-substituted 5-amino-2,6-dihydro-7\( H \)-pyrazolo[4,3-\( d \)]pyrimidin-7-ones

The compounds library was submitted to [https://www.cbligand.org/PAINS](https://www.cbligand.org/PAINS) to filter out structures (molecular weight 442-456 Da). All fragments were classified as "Accepted" due to no structural alerts and passing through the filter. All the tested compounds showed zero PAINS alert and can be used as drug compounds in the future. Theses chemical compounds will give less false positive results in high-throughput screens.\(^{430}\) There will be more specific binding with one desired target rather than nonspecific binding with numerous biological targets.\(^{431}\) Their identification will be helpful in implementing the pyrazole sub structure as an important step toward the identification of future drug candidates.

![False Positive Remover](image)

**Figure 118.** Pan Assay Interference Compounds (PAINS) results of compounds 297-298 and 301-302.
C4. 6-substituted 2-amino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-ones

Table 45. IC\textsubscript{50}s (in nM) in RFC-, PCFT- and FR-expressing cell lines and KB human tumor cells (express RFC, FR\textalpha, and PCFT) for Series XX.

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFR\textalpha</th>
<th>hFR\textbeta</th>
<th>hPCFT</th>
<th>hRFC/FR\textalpha/hPCFT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>R2</td>
<td>RT16</td>
<td>D4</td>
<td>R2/hPCFT4</td>
</tr>
<tr>
<td>300</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>6.3(1.6)</td>
<td>10(2)</td>
<td>213(28)</td>
</tr>
<tr>
<td>303</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>4.6(1.3)</td>
<td>5.6(1.4)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>304</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.58(0.12)</td>
<td>1.6(0.44)</td>
<td>23(2)</td>
</tr>
<tr>
<td>305</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>1.92</td>
<td>2.73</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PMX</td>
<td>894(93)</td>
<td>138(13)</td>
<td>42(9)</td>
<td>60(8)</td>
<td>13.2(2.4)</td>
</tr>
</tbody>
</table>

The pyrrolo[2,3-d]pyrimidine compounds 305 with 6-substitutions was designed as analog of 303 and 305. Based on the biological evaluations of 300 and 303-304 (Table 45), compound 305 was designed to evaluate and compare the effects of replacing the benzylic 11-CH\textsubscript{2} of 304 with an oxygen to compare its effects towards improving selectivity in FR\textalpha, FR\textbeta and PCFT over RFC. Compared to lead pyrrolo[2,3-d]pyrimidine analog 303, the synthesized analog 305 improve binding to FR\textalpha and Fr\textbeta by 2.5- and 2-fold, respectively (Table 41). Compound 305 had a decreased uptake through PCFT, compared to the lead compounds 303. Except for loss in PCFT activity of 305, this compound display the desired improvement in antiproliferative activity and transporter selectivity through Fr\textalpha and Fr\textbeta. To study the intracellular targets, detailed protection studies of all the synthesized compounds are currently underway.
Cell lines and assays of antitumor drug activities: The engineered CHO sublines including RFC-, PCFT- and FRα-null MTXRIIOuaR2-4 (R2), and RFC- (pC43-10), PCFT- (R2/PCFT4), or FRα- (RT16) and FRβ- (D4) expressing CHO sublines were previously described. The CHO cells were cultured in α-minimal essential medium (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), penicillin (1000 U/mL) streptomycin (1000 μg/mL) and 2 mM L-glutamine at 37°C with 5% CO₂. All the R2 transfected cells (PC43-10, RT16, R2/hPCFT4) were cultured in complete αMEM media plus 1 mg/mL G418. Prior to the cytotoxicity assays (see below), RT16 and D4 cells were cultured for 3 days in complete folate free RPMI 1640 (without added folate), plus dialyzed fetal bovine serum (FBS) (Sigma-Aldrich) and penicillin/streptomycin. KB human nasopharyngeal carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). KB cells were routinely cultured in folate-free RPMI 1640 medium, supplemented with 10% FBS (Sigma-Aldrich), penicillin-streptomycin solution, and 2 mM L-glutamine. For growth inhibition studies, cells (CHO, KB) were plated in 96 well dishes (~2500-5000 cells/well; total volume of 200 μl medium) with a range of antifolate concentrations (0-1000 nM). The experiments with RT16, D4, and KB cells used folate-free RPMI medium with 10% dialyzed FBS (Sigma-Aldrich), antibiotics and L-glutamine; the medium was supplemented with 2 nM LCV. To confirm FR-mediated drug uptake, 200 nM folic acid was added to parallel incubations. For experiments with R2, PC43-10, and R2/PCFT4 cells, cells were routinely cultured in folate free RPMI 1640 (pH 7.2)/10% dialyzed FBS with antibiotics and L-glutamine, supplemented with 25 nM LCV. Cells were incubated up to 96 h and viable cells were assayed with Cell-Titer Blue™ reagent (Promega, Madison, WI), with fluorescence measured with a fluorescence plate reader. Fluorescence data
were analyzed for calculations of IC₅₀s, corresponding to the drug concentrations that resulted in 50% loss of cell proliferation.

**FR binding assay:** To measure relative binding affinities for antifolate drugs, RT16 (expresses FRα) and D4 (FRβ) CHO cells were cultured in 60 mm dishes until they were ~80% confluent. Cells (2-4 x 10⁶ cells) were sequentially rinsed at 4 °C with Dulbecco’s phosphate buffered saline (DPBS) (3x), followed by acidic buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) (2x) (removes FR-bound folates), and finally HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose, pH 7.4) (HBS). Cells were incubated with [³H]folic acid (50 nM, specific activity 0.5 Ci/mmol) in HBS in the presence and absence of unlabeled folic acid, MTX (negative control), or the synthesized antifolates (range of concentrations up to 1000 nM) for 15 min at 0 °C. Dishes were rinsed with HBS (0-4 °C, 3x). Cells were solubilized with 0.5 N NaOH, and aliquots were measured for radioactivity and protein contents. Protein concentrations were quantified with Folin-phenol reagent. FR-bound [³H]folic acid was calculated as pmol/mg protein and binding affinities were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [³H]folic acid binding by 50%. The relative affinity of folic acid was assigned a value of 1.

**Transport assays:** R2 and R2/hPCFT4 CHO sublines were grown in suspension as spinner cultures at densities of 2-5 x 10⁵ cells/mL. Cells were collected by centrifugation, washed with DPBS, and the cell pellets (~2 x 10⁷ cells) were suspended in transport buffer (2 mL) for cellular uptake assays. PCFT-dependent uptake of 0.5 μM [³H]MTX was assayed in cell suspensions over 2 min at 37° C in HBS at pH 6.8, or in 4morpholinopropane sulfonic (MES)-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose) at pH 5.5 in the presence
of 1 or 10 µM inhibitor. At the end of the incubations, transport was quenched with ice cold DPBS, cells were washed three times with ice-cold DPBS, and cellular proteins were solubilized with 0.5 N NaOH. Levels of drug uptake were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Proteins were quantified using Folin-phenol reagent. Transport results were normalized to levels in untreated controls. Data were analyzed by Dixon plots.


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