Design and Synthesis of Pyrimidine Based Heterocycles as Potential Anti-cancer Agents with Combination Chemotherapeutic Potential or Targeted One Carbon Metabolism Inhibition and Anti-opportunistic Agents

Arpit Doshi

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DESIGN AND SYNTHESIS OF PYRIMIDINE BASED HETEROCYCLES AS POTENTIAL ANTI-CANCER AGENTS WITH COMBINATION CHEMOTHERAPEUTIC POTENTIAL OR TARGETED ONE CARBON METABOLISM INHIBITION AND ANTI-OPTHOPHUNTUNISTIC AGENTS

A Dissertation
Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Arpit Doshi

August 2019
DESIGN AND SYNTHESIS OF PYRIMIDINE BASED HETEROCYCLES AS
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ABSTRACT

DESIGN AND SYNTHESIS OF PYRIMIDINE BASED HETEROCYCLES AS
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Arpit Doshi

August 2019

Dissertation supervised by Dr. Aleem Gangjee

This dissertation describes the design, synthesis and biological evaluation of monocyclic, bicyclic and tricyclic pyrimidine-based heterocycles as a) single agents with combination chemotherapy potential having dual antiangiogenic effects and cytotoxic effects or b) one-carbon metabolism inhibitors for targeted tumor therapy; or c) selective *Pneumocystis jirovecii* (pj) dihydrofolate reductase (pjDHFR) inhibitors for pneumocystis pneumonia (PCP) infection.

The work in this dissertation is centered on identifying structural features that are necessary for inhibition of tubulin polymerization or thymidylate synthase as well as for inhibition of one or more of the receptor tyrosine kinases (RTKs)- vascular endothelial growth factor receptor-2 (VEGFR2), epidermal growth factor receptor (EGFR) and/or platelet-derived growth factor receptor β (PDGFR- β) in single entities. Single agents with both antiangiogenic activities as well as cytotoxicity (via disruption of microtubule
dynamics or thymidylate synthase inhibition) would afford agents that circumvent pharmacokinetic problems of multiple agents, avoid drug-drug interactions, could be used at lower doses to alleviate toxicity, be devoid of overlapping toxicities, and delay or prevent tumor cell resistance.

Synthesis of substituted quinazoline and pyrido[3,2-d]pyrimidines is described. It also reviews the synthesis of multi-transporter (PCFT and FR) selective 5-substituted pyrrolo[2,3-\textit{d}]/[3,2-\textit{d}]pyrimidines as targeted one-carbon metabolism enzymes (GARFTase/AICARFTase and/or SHMT2) inhibitors circumventing both dose-limiting toxicity and tumor resistance associated with most prescribed antitumor agents like pemetrexed.

PCP is a host species-specific infection. Current therapies such as trimethoprim-sulfamethoxazole (TMP-SMX) combination, which has been used for PCP for decades, has major limitations due to low inhibitory potency of TMP, side-effects of SMX and emergence of resistant strains expressing mutated dihydropteroate synthase enzyme (target of SMX). For patients unresponsive or resistant to this treatment, newer drugs are critically needed. Structure-based design, using a pjDHFR homology model and through the identification of amino acid differences between pjDHFR and hDHFR active sites, has been presented in the text. Synthesis of proposed 2,4-diaminopyrimidine analogs is presented here.

This dissertation exemplifies the use of various structural features such as a pyridyl ring, fluoro, and methyl groups as high impact medicinal chemistry design tools. The pyridyl ring has been used to introduce both conformational flexibility and restriction, whereas the combined stereoelectronic effects of the pyridyl and fluoro groups are also assessed.
DEDICATION

Dedicated to my parents and Mili
ACKNOWLEDGEMENT

I would like to acknowledge the roles of many individuals who were instrumental for the successful completion of my PhD journey. First and foremost, I am most grateful to my advisor, Professor Dr. Aleem Gangjee, for accepting me as his PhD candidate and for grooming me in an independent researcher. I am indebted to him for his scientific guidance, innovative ideas, training, and for his financial support.

I would also like to acknowledge the valuable suggestions from my committee members: Dr. Patrick T. Flaherty, Dr. Marc W. Harrold, Dr. Kevin Tidgewell, Dr. Rehana Leak, and Dr. Aleem Gangjee. I wish to express my sincere appreciation to Nancy Hosni, Mary Caruso, Jackie Farrer, Deborah Willson, and Amelia Stroyne for all their help and assistance. The acknowledgments would not be complete without mentioning all the friends I made at Duquesne: Dr. Rishabh Mohan, Dr. Manasa Ravindra, Dr. Shruti Choudhary, Dr. Khushbu Shah, Dr. Mohit Gupta, Suravi Chakrabarty, Md. Junayed Nayeen, Saloni Patel, Farhana Islam, Tasdique Quadery, Yesha Patel, Krishna Kaku, Nian Tong, Dr. Sanket Anaokar, and Harsha Pulugulla. It was such a great pleasure working with them, and I appreciate their ideas, help, and good humor. I would also like to thank the Graduate School of Pharmaceutical Sciences for financial support. I am also grateful to city of Pittsburgh where I have lived some of the most breath-taking moments of my life.

I am lucky to have inspiring, loving and supporting parents, Bharat and Surekha Doshi, who have made me what I am today and always believed in me. I cannot wish for anyone better to share my time on earth with than my wife and my best friend Mili Shah, who knows me more than anyone in the world. I am grateful to Shailesh and Kashmira, my parents-in-law, for their unconditional love. I would also like to thank Hemal, Shrushti,
Manav, Foram, Megha, Sneha, Akashi, Sachi, and Salonee, my Aunts Rekha, Chetana, Raju and Jyotsna, My Uncles Kirit, Dilip, Shailesh, and Vinod. I also want to express my gratitude to Shripali and Chinmay for accepting me as their younger brother and blessing me with their love and care. I am also lucky to have three lovely nephew and nieces, Kalp, Rahi, and Reet.
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<td>5-Amino-4-imidazole ribonucleotide synthase</td>
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<tr>
<td>AICAR</td>
<td>5-Amino-4-imidazolecarboxamide ribonucleotide</td>
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<tr>
<td>AICARFTase</td>
<td>5-Amino-4-imidazolecarboxamide ribonucleotide formyl transferase</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>AK</td>
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<td>MCT</td>
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<td>MFT</td>
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<tr>
<td>MTA</td>
<td>Microtubule Targeting Agent</td>
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<td>MTHFR</td>
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<tr>
<td>mTOR</td>
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<td>PAICS</td>
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<td>Protein Data Bank</td>
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<td>TMP</td>
<td>Trimethoprim</td>
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<td>Trimetrexate</td>
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<td>TS</td>
<td>Thymidylate Synthase</td>
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<tr>
<td><strong>VDA</strong></td>
<td>Vascular Disrupting Agent</td>
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<tr>
<td><strong>VEGF</strong></td>
<td>Vascular Endothelial Growth Factor</td>
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I. BIOCHEMICAL REVIEW

A.1 Microtubule Targeting Agents (MTAs)

Microtubule targeting agents are an essential part of cancer chemotherapy. Microtubule targeting agents target microtubule dynamics not only during mitosis but also during interphase.2, 3

A.1.1. Structure of Microtubules

![Microtubule structure and polymerization](modified from ref. 1).

Microtubules (MT) are highly dynamic intracellular polymers. The long, hollow, filamentous, tube-shaped protein polymers are essential for eukaryotic cells.4 They play crucial roles in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout cells, in cell signaling, and in cell division and mitosis. An alpha- (α) and a beta- (β) tubulin subunits form tubulin heterodimers arrange longitudinally in a polar head-to-tail fashion to form protofilaments. Thirteen protofilaments assemble laterally into tubules with a diameter of approximately 24 nm. The plus (+) end is crowned by β-tubulin and the minus (-) end is at α-tubulin (Fig. 1).5
Figure 2. Microtubule dynamics and the GTP cap (modified from ref. 1).

Guanosine-5'-triphosphate (GTP) binding and hydrolysis by αβ-tubulin dimer drives growth and shrinkage of microtubules (Fig. 2). 1 Two molecules of GTP bind to the heterodimers, and each monomer contains a GTP binding site. The α-tubulin binds irreversibly to GTP at the non-exchangeable site (N-site), and it does not hydrolyze whereas β-tubulin binds reversibly to GTP at the exchangeable site (E-site) and is prone to hydrolysis. 6 The N-site is buried within the tubulin dimer at monomer-monomer interface whereas the E-site is exposed on the surface of an unpolymerized tubulin dimer and at the plus end of the microtubules. Exchange of GDP bound on E-site with GTP renders free αβ-tubulin dimer fit for polymerization. The α-tubulin of the incoming dimer contacts the E-site on the microtubule plus end which completes the binding site that enables hydrolysis. Therefore, microtubule growth and GTP hydrolysis are coupled. Microtubules keep growing at the plus end until it has GTP-bound tubulin at the tip, once GTP hydrolysis catches up to the top of the microtubule,
rapid depolymerization and shrinkage follows. The switch from growth to shrinkage is called catastrophe, and the reverse is called rescue.

A.1.2. Binding sites of MTAs

MTAs are mainly classified into two groups based on their mechanism of action.¹

I. Microtubule stabilizing agents (MSAs): MTAs such as paclitaxel, docetaxel, epothilones, and discodermolide promote tubulin polymerization and stabilize the polymer. These compounds stabilize GDP-bound tubulin to promote microtubule polymerization.

II. Microtubule destabilizing agents (MDAs): MTAs such as Vinca alkaloids (vinblastine, vincristine, vinorelbine, vindesine and vinflunine), cryptophycins, halichondrins, estramustine, colchicine, and combretastatin promote depolymerization and prevent polymerization of tubulin are classified as microtubule destabilizing agents.

**Figure 3.** Binding sites of different classes of MTAs depicted on αβ-tubulin heterodimer conformation seen in bovine brain tubulin crystal structure (modified from ref. 7).

There are currently five structurally distinct and well-characterized sites on microtubule where drugs have been known to bind ([Fig. 3]).⁷
A.1.2.1. Taxoid binding site:

Figure 4. Taxoid binding site and representative taxanes and epothilones (modified from ref. 1).

Paclitaxel (PTX, Fig. 4), approved by FDA in 1992, was the first agent to be identified as MTA.8, 9 Along with its semisynthetic derivative, docetaxel (DTX, Fig. 4, clinically available since 1996), these drugs are used to treat a variety of solid tumors and malignancies breast cancer,10 ovarian cancer,11 nonsmall cell lung cancer,12 gastroesophageal cancer,13 germ cell tumors,14 and cancers of the head and neck.15 The binding site of PTX and its derivatives is called the taxoid site, which lies in the luminal surface of β-tubulin of the microtubule. There is a secondary site named type-I pore site (Fig. 4) next to the taxoid site.16 Majority of data on the elucidation of the binding site of MSAs suggest that they bind to either taxoid site or pore site in unassembled tubulin; while in MT they first bind to the type I pore then diffuse to the luminal taxoid site. Epothilone class of MSAs share the same binding site with the taxoids.17 Taxoid site binding agents cause restructuring of M-loop of β-tubulin, which increases the lateral tubulin interactions in microtubules, therefore, promoting the polymerization and tubulin assembly.16
A.1.2.2. Laulimalide/Peloruside Binding Site:

**Figure 5.** Structures of Laulimalide and Peloruside A (modified from ref. 1).

Laulimalide and peloruside A (Fig. 5), macrolide MTAs, bind to a non-taxane site on β-tubulin on the exterior surface of the microtubule. They bind at the interface of two adjacent β-tubulin subunits across the protofilaments. These MSAs allosterically stabilize the M-loop in the taxoid binding site.\(^\text{16}\)

A.1.2.3. Vinca-binding domain:

**Figure 6.** Vinca binding site and representative vinca alkaloids (modified from ref. 1).

The vinca alkaloids such as vinblastine and vincristine (Fig. 6) were once considered wonder drugs, which were used for various childhood hematological and solid malignancies and adult hematological malignancies.\(^\text{1}\) Other vinca derivatives like vindesine, vinorelbine, and vinflunine are in preclinical and clinical evaluation for various cancers. These drugs bind to the β-tubulin on the soluble heterodimers to a distinct region termed as a vinca-binding domain.
Other than vinca alkaloids various drugs like cryptophycins, halichondrins and dolastatins also bind at the same site.\textsuperscript{20}

**A.1.2.4. Colchicine binding site:**

![Colchicine binding site](image)

**Figure 7.** Colchicine binding site and combretastatins (modified form ref. 1).

The colchicine site binding agents include podophyllotoxin, combretastatin\textsuperscript{21} (Fig. 7), and flavonols. The binding site lies at the interface of αβ-tubulin heterodimer.\textsuperscript{22} Upon binding these agents to prevent α- and β-subunits from arranging into the straight conformation, needed for microtubule formation, due to steric clashes with α-subunit, which results in a curved conformation of the heterodimers. Therefore, they cause rapid microtubule depolymerization and mitotic arrest. CA4P (Fosbretabulin) is under extensive investigation for platinum-resistant ovarian cancer, neuroendocrine tumors (NETs), hepatocellular carcinoma, and gastric cancer.\textsuperscript{23}

**A.1.2.5. Maytansine binding site:**

![Maytansine binding site](image)

**Figure 8.** Maytansine binding site and representative binding agents.
Maytansine was shown to bind to a recently discovered new binding site on β-tubulin reported by Prota et al. Binding of maytansine to the plus end of the microtubule led to microtubule depolymerization. Maytansine is also a component of the antibody-drug conjugate Trastuzumab emtansine that was recently approved by the FDA for the treatment of HER-2 positive advanced breast cancer.

A.1.3. Mechanism of Cytotoxic Effects of MTAs

MTAs have achieved phenomenal success as cancer chemotherapy not only due to their ability to induce mitotic arrest but also due to their role in the disruption of interphase cellular mechanisms. MTAs act on both mitotic and interphase cells and their vasculature.

A.1.3.1. Mitotic cells

MTAs act on proliferating cells (cancer as well as normal cells) by decreasing microtubule dynamics, thereby arresting mitosis. On mitotic arrest, cells either die due to apoptosis or arrest in a senescence-like G1 phase. Classical MTAs such as paclitaxel and related drugs suffer from neurotoxicity and loss of neutrophils. MTAs were regarded as anti-mitotic agents for a long time, which led to development of more specific anti-proliferative agents targeting mitotic kinases and kinesin spindle protein which might lack neurotoxicity of paclitaxel. These types of inhibitors showed equipotency to MTAs in in vitro and xenograft models of cancer but failed when they reached the clinic. Also, these agents caused reversible neutropenia in patients. Doubling time, an indication of the rate of mitosis, of tumor cells in in vitro (less than 24 h) and xenograft models (1.2 to 12.2 days) is rapid whereas, in patients, majority of tumors show median doubling time of approx. 147 days. Therefore mitosis-specific antiproliferative agents failed in clinical trials, and at the same time showed toxicity against the bone marrow neutrophils, 28% of which are in mitosis at any one time. On the contrary, traditional MTAs such as taxanes, vinca alkaloids, and epothilones quickly shrink the
lesions in breast cancer, ovarian cancer, non-small-cell lung cancer, and in glioblastoma despite of slow and untimely proliferation rate of these cancer cells. This highlights that mitosis is not the only target of the MTAs.

**A.1.3.2. Interphase cells:**

![Figure 9.](image)

**Figure 9.** Diverse anticancer interphase activities of MTAs (modified from ref. 3).

An expanding body of research suggests that MTAs can cause derangement of activities of interphase cells (Fig. 9). This is because microtubules play a multitude of functions in interphase cells namely, a) centrosome clustering, b) induction of voltage-dependent anion channel opening with release of Ca²⁺ 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) impedes vesicular traffic to cell front, j) impeded timely endocytosis, k) interfere with
transcription factor transport by motors to the nucleus with 1) up- or down-regulation of tumor suppressor or oncogenes, respectively. The major toxicity of the MTAs is neurotoxicity, which is the cytotoxic effects of MSAs on non-dividing neurons in the interphase stage, which further bolsters the idea that MTAs have a mechanism of action other than anti-mitotic. Currently, it is unknown which of these effects are the most important for cell death. It is possible that MTAs act by instigating “a thousand cuts” on the cancer cells essentially living on the brink of life and death, therefore, more susceptible than normal cells.

A.1.3.3. Vascular Disrupting Effect:

Figure 10. Differences between tumor VDAs and anti-angiogenic agents (modified from ref. 33).

Tumor vasculature is an attractive chemotherapeutic target as an intact vasculature is important for growth and survival of tumors. Microtubule destabilizing agents like combretastatin have been regarded as vascular disrupting agents (VDAs). Tumor-VDAs act by selectively disrupting tumor blood vessels, inhibiting tumor blood flow, and cause extensive necrosis (Fig. 10). Selectivity for tumor vasculature over normal cells arises from differences in endothelial cells and pericytes distribution among them. There is no lymphatic drainage in tumors, and hence, the cells have higher interstitial pressure. The endothelial cells depend on microtubule to maintain cell shape. Due to the porous nature of tumor vasculature, the tumor-
VDAs can cause rapid disruption of tumor blood supply by the destruction of vascular endothelial-cadherin at the junction. This causes tumor ischemia and cell death.\textsuperscript{33}

Tumor-VDAs cause necrosis of tumor cell within the core of the tumor, whereas anti-angiogenic agents impart their activity on the tumor periphery and in small tumor masses. Due to this major difference, tumor-VDAs and anti-angiogenic agents have different toxicity and tolerability profile, offering the potential for their use on combination chemotherapy. Triple therapy of the VDA (Dimethylxanthenone-4-Acetic Acid (DMXAA)), anti-angiogenic agent (bevacizumab) and MTA (paclitaxel) caused sustained tumor regression whereas none of the single agents alone caused tumor regression.\textsuperscript{34}

\textbf{A.1.4. Resistance to MTAs}

Resistance to MTAs can occur at several stages of pharmacodynamics of the microtubule inhibition. Efflux through the membrane efflux pump of the ATP binding cassette (ABC) family is the primary mechanism of resistance towards MTAs.\textsuperscript{4} P-glycoprotein (Pgp), a product of a multidrug-resistant gene (MDR1 or ABCB1) is responsible for the efflux of MTAs. In Pgp-overexpressing SK-OV-3 MDR-1-6/6 cell line, the potency of paclitaxel was reduced 800-fold versus parental cell lines.\textsuperscript{35} Substrates of Pgp, in addition to drug efflux, cannot enter the CNS, when drugs are administered orally. Thus, agents which are less susceptible to efflux by Pgp could possess a novel pharmacodynamic and pharmacokinetic profile.

Microtubules are assembled with more than 13 diverse isotypes of $\alpha$ and $\beta$ tubulins.\textsuperscript{4} Among them, increased levels of $\beta$III-tubulin are associated with a reduced rate of responses to taxanes in tumors of the lung,\textsuperscript{36} breast\textsuperscript{37} and ovary.\textsuperscript{38} On the contrary to the effect of $\beta$III-tubulin with taxanes, epothilones are indifferent to expression of $\beta$III-tubulin.\textsuperscript{39}
A.2. Thymidylate synthase inhibitors

A.2.1. Thymidylate synthase

Figure 11. De novo synthesis of dTMP by the enzyme TS.

One of the key enzymes of folate metabolism, thymidylate synthase (TS), is present in living organisms including humans, bacteria and protozoa.\(^{40}\) It catalyzes the reductive methylation of 2'-deoxyuridine 5'-monophosphate (dUMP) to 2'-deoxythymidine 5'-monophosphate (dTMP), utilizing the coenzyme \(N^5,N^{10}\)-methylene tetrahydrofolate (5,10-CH\(_2\)THF) (Fig. 11). The dTMP is phosphorylated to 2'-deoxy-thymidine 5'-triphosphate (dTTP) by cellular kinases. The dTTP formed is utilized by deoxyribonucleic acid (DNA) polymerase and is incorporated into DNA. The TS catalyzed reaction is a key step in DNA biosynthesis and the only de novo biosynthetic pathway to dTMP.\(^{41}\) During TS catalysis, 5,10-CH\(_2\)THF acts as a one-carbon donor and is converted to dihydrofolate (DHF) (Fig. 11).

A.2.2 Structure of TS

The TS enzyme (EC 2.1.1.45) is a homodimer consisting of two identical subunits each having a molecular weight of 30–35 kDa and a primary sequence of approximately 316 amino acids.\(^{40}\) It has two active sites, each formed by residues from monomers. The primary structures of several TS enzymes, including those of humans, bacteriophages, and plants, are known. TS
enzymes are highly conserved in different species both in terms of structure and mechanism: 27 amino acids are completely conserved across all species, and 165 (80%) are conserved in more than 60% of the organisms. The active (dUMP) site of TS involves 32 amino acid residues, of which 16 are conserved in all species.

Through site-directed mutagenesis, the function of each residue in the substrate-binding site of TS has been studied. Cys198, Asn229, Arg178' and Arg179', Glu60 and Val316 (lcTS numbering) were determined to be the most important residues in dUMP binding site. During the TS catalysis, Cys198 is involved in the nucleophilic attack on C6 of the uracil ring resulting in the formation of a covalent bond between TS and dUMP. Thus no mutation is tolerated at Cys198. Asn229 (lcTS numbering), another important residue in the active site, is involved in hydrogen bonding with the 4-oxo of dUMP. Thus substitution of Asn229 by other amino acids resulted in reduced or complete loss of catalytic activity. Asn229 also plays an important role in enzyme specificity. When Asn229 is replaced with Asp229, the enzyme is no longer a deoxyuridylate methylase, but a cytidylate methylase, which catalyzes the methyltransferase reaction with 2'-deoxycytidine 5'-monophosphate (dCMP) instead of dUMP.

Amino acid residues, Arg178' and Arg179' (lcTS numbering), belong to the opposite subunit and bind to the phosphate group of the deoxyribose ring. Replacement of these two amino acids reduced the catalytic activity. The importance of other amino acids in the active site has also been studied. The C-terminal residue Val316 participates in the conformational change of the enzyme, which occurs after the covalent binding of cofactor 5,10-CH2THF to the binary complex of TS–dUMP. This conformational change is necessary for the catalytic reaction because it positions dUMP and the cofactor to facilitate the transfer of the methylene group. Pro196, Pro197, and His199 (lcTS numbering) are highly conserved amino acids, but their substitution with other amino acids is well tolerated.
A.2.3. Catalytic mechanism of TS

Figure 12. The catalytic mechanism of human TS.

The mechanism of catalysis by TS has been examined and is depicted in Fig. 12. Sequential binding of a substrate (dUMP) and cofactor 5,10-CH₂THF with TS enzyme induces a conformational change to form a non-covalent ternary complex (TS–dUMP–cofactor). In step A, Cys195 of human TS (hTS) attacks C6 of dUMP to form the enol. Protonation at the
$N^{10}$-position of 5,10-CH$_2$THF results in the formation of the reactive $N^5$-iminium species in step (B). The C5-position of the enol of dUMP reacts with the iminium ion of the activated cofactor to form an unstable covalent ternary complex in step (C). The proton at the C5-position of dUMP is abstracted by a base in the active site to provide enol in step (D). The abstraction of a proton from O4 of the enol results in the formation of exocyclic methylene and also the release of the reduced cofactor in step (E). The enzymatic reaction is completed by the hydride transfer from C6 of the reduced cofactor to form dTMP. The modified cofactor, which serves as a methylene donor and reductant, is released from the active site of TS followed by the release of the product, dTMP.

A.2.4. Inhibition of TS

Inhibition of TS depletes dTTP, one of the four building blocks required for DNA synthesis, thus leading to impaired DNA synthesis and repair. dTTP depletion also perturbs cellular levels of deoxynucleotides resulting in DNA damage. Alternatively, TS inhibition causes accumulation of dUMP and subsequent misincorporation of dUTP into DNA. These processes result in DNA double-strand breaks that lead to the initiation of apoptotic cell death, commonly referred to as “thymidine-less” cell death.

A.2.5. TS inhibitors in cancer chemotherapy

Inhibition of TS in tumor cells inhibits DNA biosynthesis resulting in thymidine-less cell death. Normal cells also require TS for DNA synthesis. Nonetheless, TS inhibitors are widely used in cancer chemotherapy, and the reason for their tumor selectivity is attributed to differences in transport, metabolism and the rates of cell division for normal and some tumor cells. As tumor cells replicate much more rapidly than normal cells, they have a higher need for a synthesis of deoxynucleotides than normal cells. Several TS inhibitors have been rationally designed to target the TS-binding site of either dUMP or cofactor 5,10-CH$_2$THF. TS inhibitors are classified broadly into two classes: dUMP-based and folate-based TS inhibitors.
A.2.5.1. dUMP-based TS inhibitors

dUMP-based TS inhibitors are antimetabolites that are analogs of dUMP, one of the substrates in TS catalysis.\textsuperscript{41} These inhibitors, upon bioactivation, either inhibit TS or are misincorporated into DNA and/or RNA resulting in the inhibition of DNA and/or RNA synthesis.

5-FU has been used for more than 50 years in the treatment of colorectal cancer.\textsuperscript{41} 5-FU remains one of the most widely used anticancer agents with a broad-spectrum activity against many solid tumors, including pancreas, breast, head and neck, gastric, and ovarian
cancers. However, 5-FU causes toxicity to normal cells because the enzymes that activate it are not tumor selective.\textsuperscript{45} For instance, 5-FU causes diarrhea because of its activation in the intestines. Moreover, it is rapidly degraded by the dihydropyrimidine dehydrogenases in the liver, thereby limiting its oral bioavailability.

Capecitabine (Xeloda\textregistered) and tegafur (\textbf{Fig. 13}) are prodrugs that are metabolized to 5-FU, which then gets converted to active nucleotides involved in DNA and RNA damage.\textsuperscript{41, 45} Rational drug design of capecitabine exploited the utilization of three tumor-overexpressing enzymes for its metabolism to 5-FU. Capecitabine is readily absorbed in the gastro-intestinal tract and is converted to 5-fluoro-5'-deoxycytidine (5-F-5'-dCR) by carboxylesterases in the liver. 5-F-5'-dCR is then converted to 5-fluoro-5'-deoxyuridine (5-F-5'-dUR) by cytidine deaminase, which is highly expressed in both liver and tumor tissues. The final step involves the conversion of 5-F-5'-dUR by either thymidine phosphorylase or uridine phosphorylase to form 5-FU. Both thymidine phosphorylase and uridine phosphorylase are highly active in tumors than in normal tissues, thus resulting in tumor specificity to capecitabine. This Medicinal Chemistry approach resulted in capecitabine with improved oral bioavailability and tumor selectivity over 5-FU. Capecitabine is used clinically in the treatment of metastatic breast and colorectal cancers.\textsuperscript{46} It has also shown promising anticancer activity in patients with prostate, pancreatic, and ovarian cancers. Tegafur (\textbf{Fig. 13}) is a prodrug that is bioactivated in the liver by cytochrome P-450 2A6 \textit{via} 5'-hydroxylation to 5-FU.

\subsection{A.2.5.1. Folate-based TS inhibitors (Antifolates)}

Antifolates are structurally related to natural cofactor 5,10-\textit{CH}_2\textit{THF} and bind to the binding site of the cofactor. These are competitive inhibitors that do not take part in the catalysis, i.e. they do not form the iminium species. The benzoyl-L-glutamate side-chain containing antifolates are called classical antifolates, where as non-classical antifolates lack the benzoyl-L-glutamate side-chain.
Figure 14. Natural cofactor 5,10-CH$_2$THF and representative antifolates as TS inhibitors.

First specific folate-based TS inhibitor was $N^{10}$-Propargyl-5,8-dideazafolate (PDDF, Fig. 14).$^{47}$ and was also the first quinazoline-containing TS inhibitor that entered phase I clinical trials. However, its development was abandoned due to its poor aqueous solubility induced renal toxicity.$^{45}$ Therefore, further derivatives containing the 2-methyl group (Raltitrexed (Tomudex®), Fig. 14) instead of a 2-amino group (PDDF) to improve water solubility and decrease renal toxicity. Raltitrexed is transported into cells via ubiquitously present reduced folate carrier (RFC) and needs to undergo polyglutamylation, via folypoly-$\gamma$-glutamate synthetase (FPGS), to increase potency (by 100-fold) and cellular retention. Raltitrexed showed good activity against advanced colorectal and breast cancers in phase II and III trials. However, it caused life-threatening toxicities, such as gastrointestinal toxicity and myelosuppression. Raltitrexed was approved as first-line therapy for the treatment of advanced colorectal cancer in several European countries, Australia, Canada, and Japan.
Clinical use of current classical antifolates is limited by resistance mechanisms such as decreased uptake by RFC and/or decreased FPGS activity.\textsuperscript{42} Nonclassical lipophilic antifolates, able to passively diffuse into cells, such as nolatrexed (Thymitaq\textsuperscript{®}) and plevitrexed (Fig. 14) have been designed to circumvent these resistance mechanisms associated with classical antifolates. Bioisosteric replacement of the $\gamma$-carboxylic acid group with tetrazole moiety in plevitrexed results into transport into cells by RFC and passive diffusion and is no more a substrate for FPGS.\textsuperscript{42} Even though; nolatrexed was the first nonclassical TS inhibitor to reach phase II clinical trials, it showed minimal activity in patients with advanced hepatocellular carcinoma.\textsuperscript{48}

Pemetrexed (\textit{Alimta\textsuperscript{®}}, \textbf{Fig. 14}) is claimed to be multtargeted antifolate that inhibits TS in addition to other folate-dependent enzymes including dihydrofolate reductase (DHFR) (very weakly), 5-aminomidazole-4-carboxamide ribonucleotide transformylase (AICARTFase), and glycinamide ribonucleotide transformylase (GARTFase) (very weakly).\textsuperscript{49} Unfortunately, it predominantly enters cells \textit{via} the RFC under normal pHs and requires polyglutamylation for maximal inhibitory effects.\textsuperscript{45} Pemetrexed is approved in the USA for the treatment of advanced nonsquamous non-small cell lung cancer and malignant pleural mesothelioma. The main toxicities associated with pemetrexed are hematologic toxicity, gastrointestinal toxicity, fatigue, and skin rashes.\textsuperscript{50}

\textbf{A.2.6. Thymidylate synthase inhibition in toxoplasmosis}

\textit{Toxoplasma gondii} (\textit{T. gondii}, \textit{tg}), an intracellular protozoan parasite, causes toxoplasmosis, a disease that affects the brain and the eyes.\textsuperscript{51} Humans are infected by ingestion of food or water that is contaminated with oocysts shed by cats or by eating raw or undercooked meat containing tissue cysts.\textsuperscript{52} According to the Centers for Disease Control and Prevention (CDC), more than 40 million people in the US have been infected with \textit{T. gondii}. While toxoplasmosis is generally benign and goes unnoticed in immunocompetent individuals, it is a
major opportunistic infection in immune-compromised patients such as those with acquired immune deficiency syndrome (AIDS) and those receiving organ transplants or chemotherapy.\textsuperscript{51} 

*\textit{T. gondii*} infection is one of the major causes of morbidity and mortality in HIV patients.\textsuperscript{53} Protozoa, including *\textit{T. gondii*}, contain a bifunctional TS–DHFR enzyme wherein the TS and DHFR exist on the same polypeptide.\textsuperscript{54} Sharma \textit{et al.}\textsuperscript{55} solved the first three-dimensional structure of *\textit{T. gondii* TS–DHFR (tgTS–DHFR) with a resolution of 3.7 Å. The tgTS–DHFR enzyme is a homodimer in which each monomer has a molecular mass of 69 kDa. Each monomer consists of a TS domain (289 residues) on the C-terminal and a DHFR domain (252 residues) on the N-terminal. TS and DHFR domains are tethered together by a linker region (69 residues). The crystal structure and the overall sequence alignment also indicated that the active site of *\textit{T. gondii* TS (tgTS) is highly conserved. Cys489 of the TS domain is involved in the catalysis of dUMP to dTMP. The key residues important for the binding to PDDF (antifolate) are Ile402, Asp513, Leu516, Phe520, Arg603 and Met608. The tgTS–DHFR exhibits substrate channeling, i.e. the dihydrofolate is directly transferred from the TS active site to the DHFR active site without entering the bulk solution. *\textit{T. gondii* relies on TS–DHFR for the synthesis of nucleotides essential for its survival. Also, *\textit{T. gondii*}, unlike humans, lacks salvage of thymidine; thus, tgTS function is necessary for survival of the parasite.\textsuperscript{54} Since the TS–DHFR enzyme is crucial for *\textit{T. gondii* survival, selective inhibition of tgTS represents a valid target to combat *\textit{T. gondii* infection. However, achieving selective inhibition of tgTS over hTS is difficult because TS is highly conserved across species (much more than DHFR) and also there is significant homology between the active site residues of tgTS and hTS as there is across species.\textsuperscript{55,56}
A.3. Receptor tyrosine kinase inhibitors

A.3.1. Angiogenesis

Figure 15. Angiogenesis in tumors (modified from ref. 58).

Angiogenesis is a process of sprouting new blood vessels from existing blood vasculature.\textsuperscript{57} Angiogenesis occurs during tumor development due to the stressful environment such as hypoxia, which forces tumors to express hypoxia-induced factors, which is primarily responsible for the recruitment of stromal cells. There is a balance between pro- and anti-angiogenic factors in normal tissues. In cancer, as the tumor grows, it should obtain its own 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 (Fig. 15).\textsuperscript{58}

The process of switching from anti-angiogenic factors to pro-angiogenic factors release is called angiogenic switching, which starts in the tumor when the tumor grows larger than 2 mm in diameter.\textsuperscript{59, 60} Tumor cells exploit their microenvironment by releasing cytokines and
growth factors. These include vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDGF) and epidermal growth factor (EGF), which promote endothelial cell proliferation/migration. Oxygen and nutrients supplied by newly infiltrated blood vessels help with tumor progression and metastases. The remodeled tumor vasculature is weak and leaky, with irregular blood flow. Therefore, tumor vasculature differs from normal tissue vasculature.

Antiangiogenic treatment is now an accepted mechanism, originally proposed by Judah Folkman approximately 48 years ago, with multiple anti-angiogenic agents approved by FDA as single agents or in combination with other cytotoxic drugs or radiation therapy. Most notable angiogenic inhibitors target receptor tyrosine kinases which are involved in the process of angiogenesis.

A.3.2. Receptor Tyrosine Kinase (RTK)

Total of 518 protein kinases have been identified in the human genome, i.e., ~1.7% of the entire genome, of which 90 belong to tyrosine kinases (TKs). Of the 90 TKs, 58 are receptor tyrosine kinases (RTKs), and 32 are non-receptor tyrosine kinases (NRTKs). NRTKs carry out intracellular communication and RTKs are involved in signal transduction. RTKs catalyze the transfer of γ-phosphate group of adenosine triphosphate (ATP) to tyrosine residues in the activation loop, a process termed as autophosphorylation. RTKs exists as monomers in an inactive form, upon ligand binding dimerization of monomers takes place. RTK monomer consists of an N-terminal extracellular ligand-binding domain, a transmembrane domain, and a C-terminal intracellular domain with tyrosine kinase activity (Fig. 16). Kinase domain is bilobar in nature with N-terminal lobe mainly made up of β-sheets and C-terminal lobe which consists of α-helices, which form a cleft at the center housing ATP binding region. ATP
binding site is divided into the adenine region, the sugar region, and the phosphate binding region.

**Figure 16.** Structure of receptor tyrosine kinase (vascular endothelial growth factor receptor (VEGFR)) and binding site of ATP (modified from refs. 69, 70, and 71).

**Figure 17.** Types of inhibitor binding sites (modified from ref 71).

The kinase domain contains a specific combination of amino acid residues aspartic acid, phenylalanine, and glycine (DFG) at the start of the activation loop in C-terminal lobe. Based on the conformation of the binding pocket and the DFG motif, RTK inhibitors are divided into four major classes (Fig. 17). ATP-competitive inhibitors that bind to the active forms of kinases with the aspartate residue of the DFG motif facing into the active site of the
kinase are called Type I inhibitors. The inhibitors that bind to the inactive forms of the kinase with the aspartate residue of the DFG motif protruding outward from the ATP-binding site of the kinase are termed as Type II inhibitors. In type III binding mode, inhibitors bind in an allosteric pocket adjacent to ATP without making any interaction with the ATP-binding pocket whereas Type IV inhibitors bind to an allosteric site remote from the ATP-binding pocket.

RTKs regulate various cellular processes such as proliferation, survival, apoptosis, metabolism, transcription, differentiation, etc. In cancers, dysregulation of RTKs creates the hallmark of cancer. Therefore, RTK inhibitors play a vital role as anticancer chemotherapy (Fig. 18).

(a) Vascular endothelial growth factor receptor (VEGFR)

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, a key regulator of blood vessel growth, binds to VEGFR-2 with $K_d$ of 75 – 125 pM. Activation of VEGFR-2 via VEGF results in signaling processes that include protein kinase activation, trans-autophosphorylation, and initiation of downstream signaling pathways, ultimately leading to angiogenesis. VEGF is expressed in a variety of cell lines derived from various hematologic malignancies, including multiple myeloma, T-cell lymphoma, acute lymphoblastic leukemia, Burkitt lymphoma, histiocytic lymphoma, and chronic myelocytic leukemia. Some examples of approved VEGFR inhibitors are bevacizumab (Avastin®) for colorectal cancer, sunitinib (Sutent®) (Fig. 18) for metastatic pancreatic neuroendocrine tumors and sorafenib (Nexavar®) (Fig. 18) for advanced renal cell carcinoma.
Figure 18. Structures of FDA-approved RTK inhibitors.

(b) Platelet-derived growth factor receptors (PDGFR)

The PDGF has four different chains—PDGF-A, PDGF-B, PDGF-C and PDGF-D, which can bind to receptors PDGFRα and PDGFRβ to activate signaling pathways such as Ras-MAPK, phosphatidylinositol 3-kinase (PI3K), and phospholipase C-γ (PLC-γ) to cause cell growth stimulation, differentiation, migration, and inhibition of apoptosis. PDGFs recruit pericytes to reduce endothelial cell proliferation and promote differentiation. PDGFR signaling complements VEGFR induced new vessel formation. Example of an approved PDGFR inhibitor is imatinib (Gleevec®) (Fig. 18) for chronic myeloid leukemia.
(c) Epidermal growth factor receptor (EGFR)

The EGF family is comprised of eleven members and four EGFR family receptors. Overexpression of EGFR leads to an increased tumor cell proliferation, survival and invasiveness. EGFR gene is an oncogene-driven gene. EGF and EGFR aid in tumor growth and metastasis via several ways: 1) increasing tumor cell proliferation and migration 2) localization of EGFR to the nucleus to promote cell proliferation 3) dysregulation of autophase activity 4) stimulation of several matrix metalloproteinases that facilitate cancer invasion and metastasis 5) EGF-mediated decrease in the abundance of mRNAs that restrain oncogenic transcription factors. Some examples of approved EGFR inhibitors are erlotinib (Tacreva®) (Fig. 18) for locally advanced or metastatic non-small cell lung cancer, gefitinib (Iressa®) (Fig. 18) for metastatic non-small cell lung cancer, and afatinib (Gilotrif®) (Fig. 18) for squamous non-small cell lung cancer.

There are two major problems with the use of RTK inhibitors as anti-angiogenic agents. First, resistance to the therapy in preclinical and clinical settings is commonly observed. This resistance is either acquired or inherent in nature. Second, severe toxicities (bleeding, hypertension, fatigue) to the treatment have been reported. In patients, resistance to anti-VEGF therapy occurs due to selection pressure on cancer cells which activates the compensatory mechanism for angiogenesis. Thus, a combination approach targeting different angiogenesis signaling could be highly effective. Additionally, a cross communication between multiple angiogenic signaling pathways can generate synergistic effects, e.g. Synergism was observed in signaling by fibroblast growth factor-2 (FGF-2) and PDGF-BB in tumor growth and metastasis. Another example of synergism is VEGF and basic fibroblast growth factor in
Many molecules function as single agents with multi-RTK inhibition potential, e.g., Sorafenib is a multi-RTK inhibitor (VEGFR-2, VEGFR-3, PDGFR-β and Rapidly Accelerated Fibrosarcoma (RAF)) used in the treatment of renal, hepatocellular and thyroid cancers. Sunitinib is a multi-RTK inhibitor (VEGFRs, PDGFRs, and c-kit) important in the treatment of renal, pancreatic, and gastrointestinal cancers. Erlotinib is an EGFR inhibitor used to treat pancreatic and non-small cell lung cancers. The success of these therapies in the clinic validates the development of single agents as multi-RTK inhibitors.

A.4. Combination chemotherapy potential in cancer

A.4.1. Combination potential of anti-angiogenic therapy with MTAs

Complex disease like cancer can only be tackled by combination therapy. The advantages attributed to a combination therapy include increased patient compliance, the emergence of an additive or synergistic interaction of the combined drugs, delay or overcome resistance, reduction of drug dosage with a consequence of diminished toxicity. Most of the tumors are heterogenic in nature. Therefore, combination therapy plays a significant role. Inhibition of a single target or pathway is known to be of limited benefit to a cancer patient due to resistance and lack of efficacy. Optimal drug combination can provide an effective therapy to combat resistance and target tumor heterogeneity.

A.4.1.1 Vascular normalization

Cytotoxic agents, such as MTAs or TS inhibitors can kill cancer cells directly, and antiangiogenic agents would kill cancer cells indirectly by depriving them of nutrients and oxygen. Clinical data has shown the success of using a combination of the cytotoxic drug with anti-angiogenic agents. The anti-angiogenic therapy instead of blocking delivery of the cytotoxic drug augments its effect. Based on this observation, Jain and coworkers formulated the “vascular normalization” hypothesis.
Figure 19. Vascular normalization hypothesis (modified from ref. 103)

As illustrated in Fig. 19, the cancer cells have haphazard blood vessels, which are leaky and tortuous.\textsuperscript{104} This creates spatial heterogeneity in blood flow, increased interstitial fluid pressure, low pH, and hypoxia.\textsuperscript{105, 106} The heterogeneity in blood flow decreases delivery of the cytotoxic drug. Acidic pH decreases the activity of immune cells and hypoxia renders cells resistant to radiotherapy.\textsuperscript{107} Anti-angiogenic therapies can stabilize vessels by increasing pericyte coverage and reduce vascular permeability/leakiness, therefore, normalizing the blood vessels.\textsuperscript{108, 109} These changes inhibit intravasation of metastatic seeking cancer cells and increase the delivery of cytotoxic drugs.\textsuperscript{103, 110, 111}

The normalization of vasculature in tumors limited to a fixed time after the anti-angiogenic therapy is administered, is defined as the “vascular normalization window” (Fig. 20).\textsuperscript{112} This window is short-lived and delivery of cytotoxic drugs in this time duration can provide a synergistic effect for the targeting the tumor.\textsuperscript{113} Lateral dosing, scheduling, sequencing, and studying the tumor vasculature is required to formulate a chemotherapy
treatment for a patient. The careful consideration is mandatory to optimize the efficacy of a combination of cytotoxic drug and anti-angiogenic agent and to reduce negative side-effects.

![Normalization window is dose and time dependent](image)

**Figure 20.** Dose and time-dependent vascular normalization window (modified from ref. 112)

Combination of tumor-VDAs with anti-angiogenic therapies is effective for targeting both core and peripheral part of the tumors (ref. section A.1.3.3).

**A.4.2 Single agents with MTA and RTK inhibitory potential**

Rationally designed single chemical entities capable of selectively targeting two or more biological targets or processes are called single agents. They are different than promiscuous drugs, which have a wide-spectrum of non-selective target activity and side-effects. Multitargeted drugs offer various advantages over combination chemotherapy, namely (i) predictable pharmacodynamic and pharmacokinetic effects; (ii) improved patient compliance; and (iii) presence of multiple entities (in one agent) with multiple effects.
A combination therapy requires a very careful selection of scheduling (Fig. 20), which is avoided while designing a single agent.

Docetaxel (MTA) plus nintedanib (Vargatef®, VEGFR, PDGFR, and FGFR inhibitor) versus docetaxel plus placebo in patients in non-small-cell lung cancer (LUME-Lung 1) phase 3 trial 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 more investigation into the combination therapy of MTAs with multi-RTK inhibitors.

![Quinazoline MTAs with multi-tyrosine kinase inhibitory activity.](image)

**Figure 21.** Quinazoline MTAs with multi-tyrosine kinase inhibitory activity.

Single agents (1a – 3a, Fig. 21) acting as MTAs along with inhibitors of VEGFR-2, PDGFRβ, and EGFR have been developed in our group previously by Shruti and Gangjee. Compounds 1a and 2a showed excellent inhibition potential across all four targets with CAM angiogenesis assay activity comparable to Sunitinib and better than Erlotinib. Separately, various scaffolds such as furo[2,3-\(d\)]pyrimidines and pyrrolo[3,2-\(d\)]pyrimidines have been shown by Gangjee and coworkers to have dual acting antitubulin and multiple RTK inhibitory activities. These studies attest to the rationale of designing compounds with anti-angiogenic and MTA potential to produce combination chemotherapy as single agents.
A.4.2 Combination potential of anti-angiogenic therapy with TS inhibition

![Chemical structure](image)

**Figure 22.** Single agents with dual RTK and TS inhibitory activity.

2,4-Diamino-thioaryl-pyrimido[4,5-b]indoles 4a and 5a (Fig. 22) have been reported by Gangjee and coworkers\textsuperscript{123} as single agents with antiangiogenic and cytotoxic activities. These compounds inhibit VEGFR-2 and PDGFR-β for antiangiogenic effects and TS for cytotoxic effects with inhibitory potencies comparable or better than the standard compounds: semaxanib (VEGFR-2 inhibitor), DMBI (PDGFR-β inhibitor) and raltitrexed (hTS inhibitor). In COLO-205 metastatic colon cancer xenograft mouse models, compound 4a significantly decreased tumor growth, liver metastases, and angiogenesis better than DMBI, remarkably without any toxicity. Antitumor activity of 5a was found to be partially due to reduced HIF-1α stabilization by increasing oxygen consumption in hypoxic conditions, which caused necrosis, reduced tumor growth, and lung metastases *in vivo*.\textsuperscript{124,125}
A.5 Folate Receptors (FR) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one-carbon metabolism inhibitors

A.5.1 Folates and its role in physiology

**Figure 23.** The chemical structures of physiologically relevant folate derivatives with different carbon oxidation states.

Water-soluble members of B9-class of vitamins are termed as folates which are involved in essential processes of growth and survival of all tissues. Folic acid can be described as the parent structure of folates (Fig. 23). The group encompasses of various substitutions attached at $N^5$ and/or $N^{10}$ of folic acid, and the presence of γ-linked glutamyl residues (2-8).
Humans cannot physiologically synthesize them and are obtained only from the ingested food. Chemically these are hydrophilic and anionic structures which cannot cross biological membranes by passive diffusion. Hence, the human body has devised several sophisticated transporter systems to absorb folic acid and its derivatives.

Figure 24. Compartmentalization of mammalian one-carbon metabolism (modified from ref. 141).

Folates are extensively involved in cellular one-carbon transfer reactions. They act as cofactors for the transfer through several of its metabolites (Fig. 24). In the cytosol, the reduced cofactor 10-formyl-THF is used in the purine biosynthesis. The enzyme glycinamide ribonucleotide formyl transferase (GARFTase) uses 10-formyl-THF for the formation of the imidazole ring of purines.

Folates are used as cofactors in several cellular one-carbon transfer reactions. In the cytosol, the reduced cofactor 10-formyl-THF is used in the purine biosynthesis by the enzyme glycinamide ribonucleotide formyl transferase (GARFTase) for the formation of the imidazole ring of purines. Downstream to the GARFTase enzyme in the purine biosynthesis pathway, 5-Aminoimidazole-4-carboxamide ribonucleotide formyl transferase
(AICARFTase) enzyme uses 10-formyl-THF to synthesize inosin-5’-monophosphate (IMP). TS catalyzes the conversion of 2′-deoxyuridine monophosphate (dUMP) to 2′-deoxothymidine monophosphate (dTMP) utilizing 5,10-CH₂-THF. The conversion generates DHF which is recycled into THF by the enzyme dihydrofolate reductase (DHFR), which is abundantly expressed in the cytosol. Another form of folic acid, 5-CH₃-THF synthesized by Methylene Tetrahydrofolate Reductase (MTHFR) from 5,10-methylene-THF in cytosol serves as a cofactor for the reaction catalyzed by Methionine Synthase (MS). MS produced methionine from cysteine, which later conjugates with Adenosine Triphosphate (ATP) to form, a methyl group donor, S-Adenosyl-Methionine (SAM). SAM causes methylation reactions on DNA, RNA, histones, and neurotransmitters, and thus can regulate transcription and translation of proteins, such as tumor suppressor genes or promoter genes.

THF cofactors can be transported into mitochondria from the cytosol via mitochondrial folate transporter (MFT/SLC25A32). In the mitochondria, it is used in the biosynthesis of formate and glycine. The intermediate folic acid derivatives can be transferred to the cytosol for various cellular synthetic reactions. Mitochondria accumulates 40% of the folate in the cell. Abnormalities in the nucleotide biosynthesis, methylation reactions, glycine biosynthesis, can affect DNA synthesis, DNA repair, DNA methylation, which can cause genomic instability of the cellular system, potentially leading to cell death.

### A.5.2 Cellular uptake of folates

Folate cofactors are hydrophilic anions at physiologic pH (7.2-7.4) and diffuse poorly across cell membranes. Consequently, folate transport systems have evolved in mammalian cells to facilitate cellular uptake. In mammalian cells, folate transport is facilitated by genetically distinct systems.
1) Reduced Folate Carrier (RFC, SLC19A1):

RFC belongs to solute carrier (SLC) family of facilitative carriers part of major facilitator superfamily (MFS) of the transporter. It is a 591- amino acid transmembrane protein with a molecular mass of approximately 85 kDa. It has 12 hydrophobic transmembrane domains (TMD) with a short hydrophilic N-terminus and long hydrophilic C-terminus. RFC is devoid of ATP-binding site domain, and thus its transport is not ATP-driven. It is a bidirectional antiporter that exchanges intracellularly stored adenosine nucleotides, thiamine monophosphate, and thiamine pyrophosphate (Fig. 25). Due to the low concentration of folates inside the cell, the transport of folates is uphill into the cell by the antiporter. It has a higher affinity for reduced folates ($K_m = 1-3 \mu M$) and lower affinity for oxidized folates like folic acid ($K_m = 200-400 \mu M$). In humans, RFC is ubiquitously
expressed in normal and malignant cells, including bone marrow, breast, lung, heart, small intestine, and lymphocytes.\textsuperscript{150-152}

2) **Proton-Coupled Folate Transporter (PCFT, SLC46A1):**

PCFT functions as a unidirectional symporter that transports folates with protons into the cells (Fig. 25).\textsuperscript{153} For instance, in the upper small intestine, the concentration of protons is high due to the activity of \( \text{Na}^+ / \text{H}^+ \) exchanger.\textsuperscript{154} Thus, the high extracellular \( \text{H}^+ \) concentration acts as the driving force for the PCFT symport into the cell. It functions optimally at pH 5.5.\textsuperscript{153} The transporter activity decreases as the pH increases. Unlike RFC, PCFT has equal affinity for both folic acid and reduced folates.\textsuperscript{153} Similar to RFC, PCFT is a MFS with 459 amino acids transmembrane protein with a molecular mass of 55 kDa.\textsuperscript{155} It has 12 TMDs and both of its N- and C-termini is in the cytosol.\textsuperscript{153} PCFT displays a 14% amino acid identity with RFC.\textsuperscript{132} No X-ray crystal structures for PCFT and RFC have been resolved so far which impedes a structure-based drug design approach.

3) **Folate receptors (FR\( \alpha \), FR\( \beta \), FR\( \gamma \)):**

FR\( \alpha \) and FR\( \beta \) are the third routes of folate influx intracellularly. These are glycoproteins with high affinity for folates and are encoded by three different loci of FR\( \alpha \), FR\( \beta \), and FR\( \gamma \). There is 70-80% amino acid homology among the FRs and have 245-257 amino acid residues.\textsuperscript{156} FR\( \alpha \) and FR\( \beta \) are glycosylphosphatidinylinositol (GPI)-anchored cell surface glycoproteins and FR\( \gamma \) is a secretory protein.\textsuperscript{156} The uptake of folates by FR\( \alpha \) and FR\( \beta \) is receptor-mediated endocytosis.\textsuperscript{157} FR\( \alpha \) is mostly expressed in epithelial cells of uterus, placenta, choroid plexus, retina, and kidney. It is also expressed in cancers of epithelial origin—adenocarcinoma of ovary, cervix, uterus, kidney, lung, breast, bladder and pancreas.\textsuperscript{156, 158, 159} FR\( \beta \) is expressed in placenta, thymus, spleen, and malignancies of myelomonocytic origin.
Figure 26. X-ray co-crystal structure of FRα with AGF183 (cyan) (PDB: 5IZQ).

X-ray crystal structures of FRα and FRβ have been resolved and published \(^{152,160}\) and the binding of folate, an image of the co-crystal structure of a known antifolate AGF183, is displayed in Fig. 26. The 2-amino-4-oxo pyrimidine motif is commonly observed across folates and antifolates. The 2-NH\(_2\) forms an ion-dipole interaction with Asp81 side chain. The 3-NH interacts with Ser174 side chain via hydrogen bonding interactions — the 4-oxo forms a hydrogen bonding interaction with the side chain of Arg103. The pyrrolo[2,3-\(d\)]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 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 Trp102 and Gln100.
The co-crystal structure of FRβ with pemetrexed has been resolved, which can be visualized in Fig. 27. The 2-amino-4-oxo pyrimidine scaffold is stabilized by π-π stacking interactions with Tyr101 and Trp187. The 2-NH₂ forms hydrogen bonding interactions with Ser190 side chain and the 4-NH₂ forms ion-dipole interaction with Asp97 side chain. The phenyl linker is also stabilized by π-π stacking interactions with Trp118. The l-glutamate 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 Trp156 side chain and backbone NH of Gly153.

A.5.3 Retention and efflux of folates

Following uptake, folates may undergo folypolyglutamylation, catalyzed by folypoly-γ-glutamate synthetase (FPGS). It catalyzes sequential addition of multiple equivalent of glutamic acid to γ-carboxyl chain of the folate cofactors. This causes an increased retention of
these polyanions intracellularly. FPGS has two forms: cytosolic and mitochondrial.\textsuperscript{162} It is also noted that polyglutamylated folates are much better substrates for various folate-dependent enzymes.\textsuperscript{163} The process of folypolyglutamylation can be reversed by enzyme $\gamma$-glutamyl hydrolase (GGH), which catalyzed hydrolysis of $\gamma$-glutamyl tail of polyglutamylated folates.\textsuperscript{164}

Several ABC transporters exist in humans. Among the multidrug resistance proteins (MRP), MRP1-5 are capable of exporting folate derivatives.\textsuperscript{165} Another exporter of folates is breast cancer resistance protein (BRCP/ABCG2).\textsuperscript{166} (Anti)Folates can get removed from the cell by overexpression of these transporters in cancer cells.

\textbf{Figure 28.} Structures of clinical antifolates

Clinically used antifolates include pemetrexed (PMX), raltitrexed, methotrexate, and pralatrexate (Fig. 28). Pemetrexed and raltitrexed are reviewed in previous section. Methotrexate is a DHFR inhibitor, which is taken up by cells via PCFT and RFC transport.\textsuperscript{133} Pralatrexate is a DHFR inhibitor with an intracellular uptake through RFC.\textsuperscript{167} It has been
approved in patients with peripheral T-cell lymphoma.\textsuperscript{167} All the clinically used folates utilize RFC transport for entry into the cell. Because of the ubiquitous expression of RFC, these compounds do not have inherent selectivity into cancer cells and cause dose-limiting toxicities (severe myelosuppression).\textsuperscript{168, 169} The process of polyglutamylation decreases when the concentration of intracellular folates (\textit{e.g.}, 5-formyl-THF) increases. Thus, activity of PMX like antifolate will decrease when concentration of intracellular folates increases. Also, PMX-resistant cancers have been identified which were developed due to the mutagenesis in the FPGS active site.\textsuperscript{170-172} Since polyglutamylation is responsible for increased potency, loss of FPGS activity can lead to drug resistant cancer cell lines.\textsuperscript{171} Several studies have displayed a reduced activity of such FPGS-dependent antifolates in cancer cells lines, which have low FPGS activity or have mutated FPGS incapable of binding to the antifolate.\textsuperscript{170,171} An increase in GGH activity also can cause decreased activity of antifolates and lead to a development of resistance.\textsuperscript{173} Overexpression of DHFR or TS, and mutations in the active site of DHFR or TS can also lead to resistance.\textsuperscript{170-172} As an approach to combat resistance and dose-limiting toxicities, there is a critical need to develop agents with (a) increased selectivity for transport \textit{via} PCFT or FRs over RFC to eradicate dose-limiting toxicities; (b) ability to target two or more intracellular enzymes to decrease development of resistance; and (c) no dependence on FPGS for its activity against intracellular targets. Dies \textit{et al.}\textsuperscript{174} and Wang \textit{et al.}\textsuperscript{175} report 6-substituted pyrrolo[2,3-\textit{d}]pyrimidines with selectivity for transport through PCFT and FRs over RFC. These compounds are potent inhibitors of GARFTase (inhibit purine synthesis). They also display a strong inhibition of GARFTase in their monoglutamylated form and do not depend on FPGS to potentiate their activity.

\textbf{A.5.4 Inhibitors of one-carbon metabolism}

\textbf{A.5.4.1 Inhibitors of \textit{de novo} purine synthesis}
Purines serve as building blocks of DNA and RNA, and as a component of ATP, cyclic adenosine monophosphate (cAMP), nicotinamide adenine dinucleotide (NAD) and coenzyme A (CoA). The synthesis can occur through de novo synthetic pathway or salvage pathway. The de novo synthetic pathway is a 10-step folate-dependent pathway (from phosphoribosyl pyrophosphate (PRPP) to IMP) (Fig. 29).

Figure 29. De novo purine nucleotide biosynthetic pathway

The enzymes catalyzing the reactions enumerated in Fig. 29 are: 1) glutamine phosphoribosylpyrophosphate amidotransferase (GPAT); 2) GAR synthase (GARS); 3) GAR formyl transferase (GARFTase); 4) formyl glycinamide ribonucleotide synthase (FGAM synthetase); 5) 5-amino-4-imidazole ribonucleotide synthase (AIRS); 6)
carboxyaminoimidazole ribonucleotide synthase (CAIRS); 7) 5-aminoimidazole-4-(N-succinylcarboxamide) ribonucleotide synthase (SAICARS); 8) adenylosuccinate lyase (ASL); 9) 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) formyl transferase (AICARFTase); and 10) IMP cyclohydrolase (ATIC). There are two steps that use 10-CHO THF and are folate-dependent in nature. They are glycinamide ribonucleotide formyl transferase (GARFTase, reaction 3) and aminimidazole carboxamide ribonucleotide formyltransferase (AICARFTase, reaction 9). The reactions 2, 3 and 5 are catalyzed by the trifunctional glycinamide ribonucleotide (GAR) formyl transferase (GARFTase), which contains GARS (reaction 2), GARFTase (reaction 3) and AIRS (reaction 5) activities. Reactions 6 and 7 are catalyzed by the bifunctional phosphoribosyl aminimidazole carboxylase/ phosphoribosyl aminimidazole succinocarboxamide synthetase (PAICS) enzyme, which contains CAIRS (reaction 6) and SAICARS; (reaction 7) activities. Reactions 9 and 10 are catalyzed by a bifunctional enzyme, 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) formyl transferase (AICARFTase)/IMP cyclohydrolase (ATIC). The two folate-dependent reactions (reactions 3 and 9) in which 10-CHO-THF serves as the one-carbon donor are catalyzed by GARFTase and AICARFTase.

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.\textsuperscript{176} Many enzymes required for salvage pathways get co-deleted in cancer cells, and cancer cells remain dependent on the de novo synthesis for obtaining purines.\textsuperscript{177} 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.\textsuperscript{178-180}
(a) GARFTase

Gangjee and coworkers have reported 6-substituted pyrrolo[2,3-\d]pyrimidines which displayed GARFTase inhibition (Table 1). Unlike PMX, which needs to be polyglutamylated for its activity, the reported compounds show potent inhibition of GARFTase in its monoglutamylated form.

Table 1. GARFTase inhibition constants (Ki)

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<thead>
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<th>n</th>
<th>Ar</th>
<th>Ki (nM)</th>
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<tbody>
<tr>
<td>AGF23</td>
<td>4</td>
<td>1,4-phenyl</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>AGF71</td>
<td>4</td>
<td>2,5-thienyl</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>AGF94</td>
<td>3</td>
<td>2,5-thienyl</td>
<td>68 ± 11</td>
</tr>
</tbody>
</table>

**Figure 30.** X-ray co-crystal structure of GARFTase and antifolate AGF150 (PDB: 4ZZ1). AGF150 is displayed in cyan color, and the substrate GAR is displayed in orange color.

The binding mode of antifolates in GARFTase can be visualized by studying the X-ray co-crystal structure of GARFTase and antifolate AGF150 (PDB: 4ZZ1, 1.35 Å) (Fig. 30).
The N1 of the pyrrolo[2,3-d]pyrimidine scaffold forms hydrogen bonding interaction with NH of the backbone of Leu899. The 2-NH$_2$ displays hydrogen bonding interaction with backbone NH of Ala947 and Glu948. The 3-NH also forms hydrogen bonding interaction with backbone NH of Ala947. The 4-oxo forms hydrogen bonding interaction with backbone NH of Asp951. The glutamate side chain is oriented in a solvent-exposed pocket — the α-COOH forms ionic bonding with Arg871 and ion-dipole interaction with backbone NH of Ile898.

(b) AICARFTase

5-Aminomidazole-4-carboxamide (AICA) and AICAR can be metabolized to AICAR monophosphate (ZMP) by adenine phosphoribosyl transferase (APRT) and adenosine kinase (AK). Thus, this can circumvent the reaction catalyzed by GARFTase. Moran and coworkers 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.

To understand the binding mode of compounds in AICARFTase, the X-ray cocrystal structure of AICARFTase with a bicyclic classical antifolate compound can be studied (Fig. 31). The bicyclic scaffold is stabilized by π-π stacking interaction with Phe544. The 2-NH$_2$ displays hydrogen bonding interaction with the backbone carbonyl of Asn489 and 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 bonding interaction with the side chain of
Figure 31. X-ray co-crystal structure of AICARFTase with bicyclic classical antifolate BW1540U88UD (PDB: 1P4R). The ligand is displayed in cyan color, and the substrate AICAR is displayed in orange color. Asn547. The sulfone in the linker shows hydrogen bonding interaction with backbone NH of Arg541, the side chain of Asn431, and ion-dipole interaction with Lys266. The glutamate side chain is oriented in a hydrophilic and solvent exposed pocket.

Figure 32. 5-substituted pyrrolo[2,3-\textit{d}]pyrimidine compounds (AGF126 and AGF127) with inhibitory activity against both GARFTase and AICARFTase. Gangjee and coworkers have reported 5-substituted pyrrolo[2,3-\textit{d}]pyrimidines (AGF126 and AGF127) which displayed inhibition of both GARFTase and AICARFTase (Fig. 32). This dual inhibition is advantageous as it attacks the purine biosynthetic pathway at
two distinct sites and could overcome a tumor resistance due to lack of sensitivity at a single target. Such therapy will be effective for drug-resistant tumors.

A.5.4.2 Inhibitors of serine hydroxymethyl transferase enzyme

![Figure 33. Compartmentalization of 1C metabolism (modified from ref. 126).](image-url)

One carbon units incorporated in various folate metabolism cofactors are primarily derived from serine catabolism occurring in mitochondria.\(^{126,186}\) Therefore, serine catabolism plays essential role in cancer cell growth. Serine is catabolized in mitochondria and synthesized in cytosol by set of enzymes which allow not only for parallel metabolic processes, but also for a complete oxidative/reductive cycle.\(^{126}\) In mitochondria, serine is metabolized to formate via three enzymes: a) serine hydroxymethyl transferase 2 (SHMT2), b) methylene tetrahydrofolate dehydrogenase 2/L, and c) monofunctional tetrahydrofolate synthase (MTHFD1L) whereas serine is synthesized in cytosol via a) methylene tetrahydrofolate dehydrogenase, and formyltetrahydrofolate synthetase 1 (MTHFD1), and b) serine hydroxymethyl transferase 1 (SHMT1). The cycle that catabolizes serine in mitochondria and synthesizes serine in the cytosol is thermodynamically driven by the difference in electrochemical potential between mitochondrial NADH (NADPH) and cytosolic NADPH.
In rapidly proliferating cells such as cancer cells there is continuous need for 1C units, which results in consistent overexpression of SHMT2 and immediately downstream enzyme MTHFD2 in most of the cancers.\textsuperscript{187-189} The 1C unit generated from serine catabolism in the mitochondria is exported to cytosol as formate, which is incorporated in THF and subsequently by series of reactions catalyzed by GARFTase, AICARFTase and thymidylate synthase used for nucleotide synthesis.\textsuperscript{141, 190, 191} Even though SMHT2 and MTHFD2 deleted cells are viable, primarily due to 1C pool compensation by the cytosolic SHMT1 enzyme.\textsuperscript{191, 192} However, the flux carried through this enzyme is not sufficient to meet glycine demand and these cells are glycine auxotrophs.\textsuperscript{126} This lack of glycine becomes more pronounced in cancer cells which are not able to access serum glycine supply and rely primarily on intracellular synthesis.\textsuperscript{193} To achieve cytotoxic effect simultaneous inhibition of both cytosolic and mitochondrial SHMT enzymes is necessary. Khushbu and Gangjee\textsuperscript{194} have recently reported novel pyrrolo[3,2-$d$]pyrimidine antifolates which inhibit both SHMT1 and 2 enzyme along with enzymes in \textit{de novo} purine biosynthesis pathway and are transported selectively into the tumor cells. This resulted in excellent \textit{in vivo} efficacy in pancreatic cancer model. These compounds are exciting prototypes for dual-targeting mitochondrial and cytosolic C1 metabolism for cancer, with substantial promise for overcoming resistance to current anticancer therapies.

\textbf{A.6. Selective pjDHFR inhibitors}

\textbf{A.6.1. \textit{Pneumocystis jirovecii} and pneumocystis pneumonia Infection}

\textit{Pneumocystis jirovecii} (pj) is a fungus which is present in the lungs of a majority of the human population around the world.\textsuperscript{195} It is an atypical fungus, which differs from other fungi due to the presence of cholesterol, instead of ergosterol, in its cell membrane. The species affecting humans is very different than the species affecting other animals, such as rats and mice. The species that infects rats does not proliferate when passaged into mice, whereas the infection from rats to other rats causes severe infection.\textsuperscript{196} The immune system in healthy
individuals keeps the *Pneumocystis jirovecii* infection under control. In immunocompromised patients, *Pneumocystis jirovecii* infection causes Pneumocystis pneumonia (PCP). PCP can be fatal for patients with HIV/AIDS (most common), patients undergoing chemotherapy for cancer, patients on immunosuppressive medications, patients undergoing organ or bone-marrow transplantation or those that are malnourished.\textsuperscript{195,197} PCP presents itself when the patients’ CD4 count is below 200 cells/mm\textsuperscript{3}. Although PCP prophylaxis and antiretroviral therapy (ART) has changed the face of the HIV/AIDS epidemic, the incidences of HIV cases persist due to non-adherence 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.\textsuperscript{198,199} Thus, PCP continues to be a significant public health burden. In the US, 9\% of the hospitalized HIV/AIDS and 1\% of organ transplant patients develop PCP infection.\textsuperscript{200} In these patients, the mortality rate is from 5-40\% while being treated for PCP and approaches 100\% if left untreated.\textsuperscript{200}

Both the prophylaxis and treatment for PCP involves the combination of trimethoprim (TMP)-sulfamethoxazole (SMX) (co-trimoxazole).\textsuperscript{201,202} TMP (Fig. 34) is a selective, but weak inhibitor of dihydrofolate reductase (DHFR), the enzyme necessary for the reduction of dihydrofolate to tetrahydrofolate,\textsuperscript{203} while SMX is an inhibitor of the dihydropteroate synthase (DHPS), the enzyme necessary for the synthesis of folates in fungi.\textsuperscript{204} The low activity of TMP against DHFR is augmented by SMX, in the treatment regimen. The efficacy, low cost and activity in variety of infections has propelled cotrimoxazole to be used indiscriminately. Due to the rampant use, mutations in the DHPS locus of *Pneumocystis jirovecii* encoding DHPS have been documented as the cause of TMP/SMX resistant strains of PCP.\textsuperscript{204-207}
Various studies have also reported clinical and non-clinical mutations discovered in DHFR after treatment or prophylaxis using DHFR inhibitors.\textsuperscript{208-212} Treatment failure and discontinuation of co-trimoxazole occurs in several cases due to such resistant strains or toxicity/allergy by SMX.\textsuperscript{213, 214} In cases of treatment failure to TMP/SMX, the second-line treatment in mild to moderate PCP is TMP-dapsone or clindamycin-primaquine, which also leads to low efficacy and often lethal side-effects.\textsuperscript{201, 215, 216} Piritrexim (PTX) and trimetrexate (TMQ) are potent, but non-selective inhibitors of DHFR, which cause dose-limiting toxicities.\textsuperscript{201, 217, 218} These agents, combined with leucovorin (for rescue from myelosuppression) had high costs, drug toxicities, drug interactions and lack of efficacy. For patients that do not respond to first line treatment as well the inevitable recurrence of resistance, new drugs for the treatment of PCP are critically needed.

**Figure 34.** Clinical and non-clinical agents for the treatment for PCP
A.6.2. DHFR

Dihydrofolate reductase (DHFR) is an important target for several human diseases—protozoal, bacterial, fungal infections, and autoimmune diseases.\textsuperscript{219,220} Folate metabolism has been investigated and validated for decades as a target in chemotherapy. Eukaryotic organisms synthesize thymidine \textit{via} thymidylate cycle, which consists of enzymes: (a) Serine hydroxymethyl transferase (SHMT); (b) DHFR; and (c) Thymidylate synthase (TS). DHFR catalyzes NADPH-dependent reduction of 7,8-dihydrofolate to the 5,6,7,8-tetrahydrofolate (THF) (Fig. 35). The THF then acts as a cofactor in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), catalyzed by TS. This inhibition causes disruption in DNA, RNA and protein synthesis of the organism and eventually leads to death. Thus, inhibition of TS or DHFR causes a “thymineless death” and this concept has been proven for several antimalarial, antiprotozoal and antimicrobial agents.\textsuperscript{203}

\textbf{Figure 35.} Reaction catalyzed by DHFR\textsuperscript{219}
A.6.3. hDHFR, pcDHFR and pjDHFR

Pneumocystis infection is host specific. Most of the drugs synthesized and evaluated thus far for PCP infections were tested against *Pneumocystis carinii* DHFR (pcDHFR), which was presumed to be the causative species of PCP infection in humans.\(^{203}\) *Pneumocystis carinii* (pc) however is a distinct species that infects rats, different from *Pneumocystis jirovecii*, responsible for human infections. The amino acid sequence of the DHFR of *Pneumocystis carinii* (pcDHFR) differs by 38% when compared to the DHFR of *Pneumocystis jirovecii* (pjDHFR).\(^{221}\) Hence, drugs evaluated against the surrogate pcDHFR in vitro, may not translate into activity in the treatment of PCP infection in humans.\(^{222}\) Rational design of pjDHFR inhibitors is hampered due to a lack of crystal structure information for pjDHFR. Another significant impediment in the drug discovery of inhibitors of pjDHFR is the inability to grow the organism outside the human lung and hence to develop a tissue culture for in vitro studies or an animal model for in vivo evaluation of the synthesized compounds.

**Figure 36.** Previously reported pyrido[2,3-\(d\)]pyrimidines.

Gangjee and coworkers\(^{222}\) reported pyrido[2,3-\(d\)]pyrimidines (Fig. 36) as selective and potent pjDHFR inhibitors (Table 2). To illustrate the interactions that defines the enhanced selectivity and potency, Cody *et al.*\(^{223}\) reported the X-ray co-crystal structures of 8a with pcDHFR and hDHFR (PDB: 4IXE and 4QJC, respectively).
Table 2. Inhibitory Concentrations (IC$_{50}$, in nM) against Recombinant DHFR from pjDHFR and human DHFR (hDHFR) and Selectivity Ratios.\textsuperscript{211}

<table>
<thead>
<tr>
<th></th>
<th>pjDHFR (IC$_{50}$, in nM)</th>
<th>hDHFR (IC$_{50}$, in nM)</th>
<th>h/pj</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>2.4</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>7a</td>
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<td>8a</td>
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<td>TMP</td>
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<tr>
<td>TMQ</td>
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Figure 37. 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.
The variability in pcDHFR, pjDHFR and hDHFR enzyme sequences have been investigated by Cody et al.\textsuperscript{212} The particular residues which seem to influence the active site (as seen in Fig. 37) are at positions 35 and 64 (hDHFR numbering). In 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 these residues interact with inhibitors and that inhibitors have been designed in the past to specifically target these residues.\textsuperscript{47, 211, 220}

TMP is a moderate pcDHFR and pjDHFR inhibitor. The reason for its safety in PCP infection is its inactivity against hDHFR (Table 2). The importance of the positions 35 and 64 in hDHFR was illustrated by evaluating the Ki of TMP against wild type and mutants of hDHFR (Table 3). The Q35S mutant hDHFR displays 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, selectivity of agents can be achieved for pjDHFR over hDHFR by rational design of compounds targeting the amino acid residues that are different in hDHFR and pjDHFR enzymes.

\textbf{Table 3.} Kinetic constants (Ki) of TMP against pjDHFR, pcDHFR, and wild type and mutant hDHFR.\textsuperscript{212}

<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>
II. CHEMICAL REVIEW

The chemistry related to the present work is reviewed and includes synthetic approaches to the following heterocyclic scaffolds.

B.1. Quinazolines

B.2. Pyrido[3,2-\textit{d}]pyrimidines

B.3. Pyrimido[4,5-\textit{b}]indoles

B.4. Pyrrolo[2,3-\textit{d}]pyrimidines

B.5. Pyrrolo[3,2-\textit{d}]pyrimidines

B.1. Quinazolines

Various synthetic methodologies have been employed in the synthesis of quinazolines. Few of them will be discussed here from substituted benzene precursors.

\textbf{Scheme 1.} Synthesis of quinazoline from anilines

Protection of the amino group of the aniline 1b with ethyl chloroformate (Scheme 1) and subsequent reaction with hexamethylenetetramine (HMTA) in trifluoroacetic acid (TFA) resulted in the formation of cyclized dihydrobenzoquinazoline 3b. Oxidation of 3b using \( \text{K}_3\text{Fe(CN)}_6 \) afforded the appropriate quinazoline 4b in 15-54% yield.\textsuperscript{224,225}
Scheme 2. Synthesis of quinazoline from 2-aminoarylketones and benzylamines

Graphite oxide was used as a recyclable heterogenous catalyst (Scheme 2) in the synthesis of 2-arylinazoline 7b (84-93%), from 2-aminoarylketones 5b and benzylamines 6b. To ensure complete oxidation t-butyl hydroperoxide (TBHP) was used.225,226

Scheme 3. Synthesis of quinazoline from 2-iodobenzaldehydes via Ullmann reaction

Ligand-free Cu-catalyzed Ullmann reaction conditions (Scheme 3) were used for preparing substituted quinazolines 10b in 61-89% yield from 2-iodobenzaldehydes 8b and amidine hydrochloride 9b.225,227

Scheme 4. Synthesis of 2-aryl-4-methylquinazolines

In a similar reaction, quinazolines 13b (Scheme 4) were synthesized substituted 2-bromoacetophenone 11b, aldehyde 12b and aq. the in the presence of Cu catalyst (CuI) and
N-methylpyrrolidone (NMP) as a solvent. The reaction was proposed to occur via Cu(I) catalyzed amination of 11b to yield substituted 2-aminobenzophenone that further reacted with the aldehyde 24. 225, 228

**Scheme 5.** Synthesis of 1(\(H\))-quinazolin-2-one from alkynylanilines

The synthesis of target compounds was accomplished in two steps (Scheme 5). In the first step, the urea derivative, 15b was obtained by reacting o-alkynylaniline 14b with potassium isocyanate. In the subsequent step, acid catalyzed intramolecular cyclization of 15b yielded 16b in 28-98% yield. 225, 229

**Scheme 6.** Synthesis of quinazolin-4(3\(H\))-one from anthranilic acid

Treatment of anthranilic acid 17b (Scheme 6) with aliphatic anhydride yielded corresponding benzoxazinones 18b, which were used further without purification to form acylaminobenzamides 19b. Subsequent cyclization with formamide under microwave
irradiation conditions afforded the desired quinazolin-4-one 20b in 74-87% yield.\textsuperscript{225,230}

In another method, 2-nitrobenzamide 33 was reacted with aromatic aldehyde 34 in the presence of sodium dithionate to yield quinazolin-4(3\textit{H})-one 26b (Scheme 7). Mechanistically, the reaction proceeded via reduction of nitro by sodium dithionate to amine 23b that cyclized with the aldehyde to yield 2,3-dihydroquinazolin-4(1\textit{H})-one 24b. The sulfur dioxide from sodium dithionate subsequently participated in the oxidation to yield desired compound 26b.\textsuperscript{225,231}

\textbf{Scheme 7.} Synthesis of quinazolin-4(3\textit{H})-one from 2-nitrobenzamides

\begin{align*}
\text{Ar} & \text{OH} + \text{R}^1\text{CHO} \rightarrow \text{R}^1\text{CONH} \quad \text{Na}_2\text{S}_2\text{O}_4 \\
\text{21b} & \text{22b} \quad \text{DMF/H}_2\text{O} 9:1 \\
& 90 \degree \text{C} \\
\text{Na}_2\text{S}_2\text{O}_4 & \text{DMF/H}_2\text{O} \\
\text{R}^1\text{CONH} & \text{NH} \quad \text{R}^1 \\
\text{23b} & \text{24b} \quad \text{SO}_2 \\
\text{SO}_2 & \text{25b} \quad \text{SO}_2
\end{align*}

\textbf{Scheme 8.} Synthesis of quinazolin-4(3\textit{H})-one from isatoic anhydride

\begin{align*}
\text{Ar} & \text{X} + \text{R-NH}_2 + \text{29b} \rightarrow \text{30b} \\
\text{27b} & \text{28b} & \text{29b} \quad \text{K}_2\text{CO}_3 \\
& \text{DMSO} \quad \text{(90-97 \%) 17 examples}
\end{align*}

In a one-pot three-component reaction (Scheme 8) with benzyl halides 27b, amines 28b and isatoic anhydride 29b quinazolin-4(3\textit{H})-one 30b were synthesized \textit{via in situ}
generations of aldehydes by DMSO mediated oxidation of benzyl halide in the presence of K$_2$CO$_3$ in excellent yields.$^{225,232}$

**B.2. Pyrido[3,2-\textit{d}]pyrimdines**

**Scheme 9.** Synthesis of 6-chloropyrido[3,2-\textit{d}]pyrimidine-2,4-diamine from 2,6-dichloropyridine

The 2,4-diamino-6-chloropyrido[3,2-\textit{d}]pyrimidine 35b (Scheme 9), was obtained from 2,6-dichloropyridine 31b via a four-step synthetic procedure.$^{233}$ The 2,6-dichloropyridine was nitrated with 90% nitric acid and sulfuric acid to afford 32b. This was followed by substitution of the 2-Cl moiety with cuprous cyanide at 180 °C to afford 2-cyano-3-nitro-6-chloropyridine 33b. Following the reduction of 33b with iron powder in acidic methanol to afford the 3-amino-6-chloropicolinonitrile 34b. The 2-cyano-3-amino function was set up to condense with chloroformamidine hydrochloride in dimethyl sulfoxide to afford the crucial intermediate 35b (85% over four steps).

Wang et al.$^{234}$ have reported synthesis of (\textit{E})-6-styrylpyrido[3,2-\textit{d}]pyrimidine-2,4-diamine derivative 41b from 5-aminopyrimidine-2,4-diol 36b (Scheme 10). Commercially available 5-aminouracil 36b and crotonaldehyde were cyclized in 20% HCl using the Skraup reaction to give 2,4-dihydroxy-6-methylpyrido[3,2-\textit{d}]pyrimidine 38b. The 6-methylpyrido[3,2-\textit{d}]pyrimidine 38b was then reacted with substituted benzaldehyde in the
presence of p-toluenesulfonamide using \(N,N\)-dimethylacetamide as a solvent to form \((E)\)-2,4-dihydroxy-6-phenylethynylpyrido[3,2-\(d\)]pyrimidine \(39b\). Chlorination of intermediate \(39b\) with an excess of \(\text{POCl}_3\) in the presence of catalytic pyridine provided the 2,4-dichloro derivative \(40b\). Conversion of \(40b\) to the corresponding 2,4-diamino derivative \(41b\) was achieved using a saturated solution of ammonia in dry methanol in a sealed vessel at 150 °C for 8 h.

**Scheme 10.** Synthesis of \((E)\)-6-styrylpyrido[3,2-\(d\)]pyrimidine-2,4-diamine from 5-aminopyrimidine-2,4-diol

Mamouni *et al.*\(^\text{235}\) reported a four-step procedure for synthesis of pyrido[3,2-\(d\)]pyrimidine-2,4-diones from furo[3,4-\(b\)]pyridine-5,7-dione (**Scheme 11**). The esterification of pyridine-2,3-dicarboxylic anhydride \(42b\) by methanol afforded a mixture of \(43b\) (stable isomer) as a major product and \(44b\) (unstable isomer) as a minor product (**Scheme 11**). Treatment of 2-(methoxycarbonyl)nicotinic acid \(43b\) by ethyl chloroformate with triethylamine and sodium azide in the presence of a small amount of water gave the methyl 3-
(azidocarbonyl)picolinate 45b which was transformed by Curtius rearrangement into methyl 3-isocyanoatopicolinate 46b. The subsequent reaction of isocyanate 46b with amino acids under mild conditions by gentle heating led to pyrido[3,2-\(d\)]pyrimidinediones 47b-49b.

**Scheme 11.** Synthesis of pyrido[3,2-\(d\)]pyrimidine-2,4-diones from furo[3,4-\(b\)]pyridine-5,7-dione

![Scheme 11](image)

**Scheme 12.** Synthesis of 6-aryl-1,3-dimethyl-8-phenylpyrido[3,2-\(d\)]pyrimidine-2,4(1\(H,3H\))-dione from 5-amino-1,3-dimethylpyrimidine-2,4(1\(H,3H\))-dione

![Scheme 12](image)

Majmudar *et al.*\(^{236}\) devised a mild and efficient method for the synthesis of pyrido[3,2-\(d\)]pyrimidine derivatives 53b-56b (Scheme 12) via three-component domino reaction of
amines, aldehydes, and terminal inactivated alkynes. This method used BF$_3$·OEt$_2$ as a Lewis acid catalyst in one pot.

**B.3. Pyrimido[4,5-b]indoles**

![Chemical structure of pyrimido[4,5-b]indoles](image)

**Figure 38.** Disconnection strategy for pyrimido[4,5-b]indoles from indole precursors.

Different methods used in the synthesis of pyrimido[4,5-b]indoles from indoles or tetrahydroindoles utilize a disconnection strategy, as shown in Fig. 38.

**Scheme 13.** Synthesis of 2-amino-pyrimido[4,5-b]indoles from indoles

![Synthesis of 2-amino-pyrimido[4,5-b]indoles from indoles](image)

Treatment of 1,2-dichloro-3-nitrobenzene **57b** with ethyl cyanoacetate provided **58b**, which underwent reductive cyclization to the indole **59b** (Scheme 13). Cyclocondensation of **59b** with chlorformamidine hydrochloride afforded 2-amino-4-oxo-5-chloro-pyrimido[4,5-b]indole **60b**.
Alternatively, 2-amino-4-oxo-pyrimido[4,5-b]indoles can be synthesized by a two-step procedure as shown in Scheme 14 below.\textsuperscript{237}

**Scheme 14.** Sequential two-step synthesis of 2-amino-pyrimido[4,5-b]indoles

![Chemical structure](image1)

**Scheme 15.** Synthesis of the 2-unsubstituted pyrimido[4,5-b]indole 67b.

![Chemical structure](image2)

Analogous to 4-chloroindole derivative 59b in Scheme 13, the 5-chloroindole 66b (Scheme 15) was obtained from 1,3-dichloro-4-nitrobenzene 64b.\textsuperscript{238} Compound 66b was
cylized using formamide to afford 2-unsubstituted 4-oxo-6-chloro-pyrimido[4,5-b]indole 67b.

Scheme 16. Versatile synthesis of 2-substituted pyrimido[4,5-b]indoles

Showalter et al.\textsuperscript{239} reported a versatile procedure for the preparation of 2-substituted 4-oxo-pyrimido[4,5-b]indoles 69b, 72b and 73b from ethyl 2-amino-1H-indole-3-carboxylate 68b (Scheme 16). Compound 68b was cyclized using formamide under basic conditions to 2-unsubstituted pyrimido[4,5-b]indole 69b. Treatment of 68b with cyanamide and acetonitrile under acidic conditions provided intermediates 70b and 71b, which were cyclized using 1N NaOH to 2-amino- and 2-methyl-pyrimido[4,5-b]indoles 72b and 73b, respectively.

Venugopalan et al.\textsuperscript{237} reported the synthesis of 2-substituted pyrimido[4,5-b]indoles from ethyl 2-amino-4,5-dimethoxy-indole carboxylate 61b (Scheme 17). Treatment of 61b with ethyl carboxyisothiocyanate gave 74b, which cyclized to the 2-thio-4-oxopyrimido[4,5-b]indole 75b under basic conditions. Methylation of 75b afforded the 2-methylthio-pyrimido[4,5-b]indole 76b, which upon reduction gave the 2-unsubstituted pyrimidoindole 77b. For the synthesis of 2-unsubstituted pyrimido[4,5-b]indoles, this strategy presents an alternative to the approach described in Schemes 15 and 16, in which 2-unsubstituted
pyrimidoindoles 67b (Scheme 15) and 69b (Scheme 16) were synthesized by a direct reaction of indoles with formamide.

Scheme 17. Venugopalan strategy for the synthesis of 2-substituted pyrimido[4,5-b]indoles

Kumar et al. synthesized pyrimido[4,5-b]indoles from 2-haloindoles via palladium-catalyzed amidation and cyclization. Coupling of the 3-formyl- or 3-acetyl-2-haloindoles 78b and amides 79b provided 80b in 80–94% yield (Scheme 18). Treatment of 80b with ammonium formate resulted in the formation of pyrimido[4,5-b]indoles 81b. According to the proposed mechanism for 80b→81b, ammonium formate thermally decomposes and releases ammonia which attacks 3-carbonyl of 80b and generates the “imine” intermediate, which then cyclizes to provide the desired pyrimido[4,5-b]indole 81b.

In Schemes 13–18 above, indoles were utilized to obtain pyrimido[4,5-b]indoles.

Schemes 19 and 20 below present synthesis of pyrimido[4,5-b]indoles from tetrahydroindoles.

Scheme 19. Synthesis of pyrimido[4,5-b]indoles from tetrahydroindoles

The tetrahydro-1H-indole 83b (Scheme 19) was obtained by the treatment of 2-hydroxycyclohexanone 82b with benzylamine and malononitrile. Compound 83b was
cyclized using formic acid to the 4-oxo-tetrahydropyrimido[4,5-b]indole 84b. Chlorination of the 4-oxo of 84b followed by displacement with 3-chloroaniline afforded 85b. Oxidation of 85b using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and subsequent removal of the benzyl group provided pyrimido[4,5-b]indole 86b.

Müller et al. synthesized a series of chiral pyrimido[4,5-b]indole derivatives 91b and 92b (Scheme 20). Reaction of chiral tetrahydroindoles 87b with benzonitrile and sodium methoxide yielded the 2-phenyl-4-amino-tetrahydropyrimido[4,5-b]indoles 88b. On the other hand, treatment of 87b with benzoyl chloride provided 90b, which was subsequently cyclized to the 2-phenyl-4-oxo-tetrahydropyrimido[4,5-b]indoles 91b. Dehydrogenation of the tetrahydroindoles 88b and 91b furnished the desired pyrimido[4,5-b]indoles 89b and 92b, respectively.

**Scheme 20.** Synthesis of 2-phenyl-pyrimido[4,5-b]indoles from tetrahydroindoles

\[ R = (R)\text{-} \text{or} \ (S)\text{-}1\text{-phenylethyl} \]
\[ (R)\text{-} \text{or} \ (S)\text{-}1\text{-}(4\text{-}methylphenylethyl) \]
\[ (R)\text{-} \text{or} \ (S)\text{-}1\text{-methyl-2-phenylethyl} \]
B.4. Pyrrolo[2,3-\textit{d}]pyrimidines

Scheme 21. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from 6-aminopyrimidin-4(3\textit{H})-one

6-amino-5-bromopyrimidin-2(1\textit{H})-one, \textit{94b} (Scheme 21) was synthesized by electrophilic substitution of 6-aminopyrimidin-2(1\textit{H})-one \textit{93b} with bromine. Palladium catalyzed Stille coupling of \textit{94b} with Z-1-ethoxy-2-(nbutylstanyl)ethane followed by deprotection of resulting enol ether \textit{95b} with concomitant cyclization gave 4-methyl-1,3,4,7-tetrahydro-2\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidin-2-one, \textit{96b}.243

Scheme 22. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from ethyl 2-cyano-4,4-diethoxybutanoate

Cyclocondensation of ethyl 2-cyano-4,4-diethoxybutanoate \textit{97b} (Scheme 22) with thiourea affords 6-amino-5-(2,2-diethoxyethyl)-2-thioxo-2,3-dihydropyrimidin-4(1\textit{H})-one, \textit{98b}. Acidic hydrolysis of acetal \textit{98b} and cyclization and final desulfurization with Raney nickel forms 1,2,3,7-tetrahydro-4\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidin-4-one, \textit{100b}.243
Scheme 23. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from 5-bromo-2,4-dichloropyrimidine

The synthetic route (Scheme 23), based on Besong’s method\textsuperscript{244} starts with the reaction of 5-bromo-2,4-dichloropyrimidine, 101\textit{b} and cyclopentylamine in \textit{N},\textit{N}-diisopropylethylamine to yield compound 102\textit{b}, followed by Pd catalyzed Sonogashira coupling with propargyl alcohol afforded 3-(2-chloro-4-(cyclopentylamino)pyrimidin-5-yl)prop-2-yn-1-ol, 103\textit{b}. Cyclization of 103\textit{b} was carried out in THF using TBAF to afford (2-chloro-7-cyclopentyl-7\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidin-6-yl)methanol, 104\textit{b}.\textsuperscript{245}

Scheme 24. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines from 6-amino-2-(dimethylamino)pyrimidin-4-(3\textit{H})-one

Gangjee \textit{et al.}\textsuperscript{246} reported the synthesis of 109\textit{b} (Scheme 24) by the condensation of an aqueous solution of \(\alpha\)-chloroacetone 105\textit{b} in presence of NaOAc in 70% yield. Linz \textit{et al.}\textsuperscript{247} 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 106\textit{b} (Scheme 24). Compound 106\textit{b} on treatment with
α-chloroacetone 107b or α-chloroacetaldehyde 108b provided the corresponding pyrrolo[2,3-d]pyrimidines 109b or 110b in 68% and 75% yields respectively.

Taylor et al.\textsuperscript{248} synthesized methyl 4-(2-(2-amino-6-methyl-4-oxo-4,7-dihydro-3Hpyrrolo[2,3-d]pyrimidin-5-yl)ethyl)benzoate 113b (Scheme 25) by a Fisher-Indole approach. Reaction of 2-amino-6-hydrazinylpyrimidin-4(3H)-one, 111b with one equivalent of methyl 4-(4-oxopentyl)benzoate in 2-methoxyethanol under reflux afforded the requisite intermediate 112b, methyl (Z)-4-(4-(2-(2-amino-6-oxo-1,6-dihydropyrimidin-4-yl)hydrazineylidene)pentyl)benzoate. The cyclization was accomplished by thermolysis of 112b in refluxing diphenyl ether to regioselectively afford the pyrrolo[2,3-d]pyrimidine 113b.

**Scheme 25.** Synthesis of pyrrolo[2,3-d]pyrimidines using Fisher-Indole reaction

![Scheme 25](image)

**Scheme 26.** Synthesis of pyrrolo[2,3-d]pyrimidines from 1-benzyl-2-amino-3-cyanopyrroles

![Scheme 26](image)

Traxler et al.\textsuperscript{241} reported the synthesis of a series of pyrrolo[2,3-d]pyrimidines (Scheme 26) starting from substituted α-hydroxy ketone 114b. Ketone 114b was converted to 2-amino-
1-benzyl-4,5-dimethyl-1H-pyrrole-3-carbonitrile 115b by treatment with benzylamine at reflux in toluene and further condensation with malononitrile in toluene at reflux. 2-Amino-1-benzyl-4,5-dimethyl-1H-pyrrole-3-carbonitrile, 115b underwent condensation with 85% formic acid at reflux to provide the target 7-benzyl-6-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-ol, 116b.

**Scheme 27.** Synthesis of 2-amino-pyrrolo[2,3-d]pyrimidines from 4-substituted 2,6-diamino pyrimidines

Khalaf *et al.* synthesized pyrrolopyrimidines 124b-128b (Scheme 27) with 5-hydrophobic substituents via Michael addition-based synthesis to include a wide range of aryl and some alkyl substituents in both 4-oxo- and 4-aminopyrimidine series. Using 4- substituted 2,6-diamino pyrimidines 117b with (E)-(2-nitrovinyl)aryl 118 to afford 5-(1-aryl-2-nitroethyl)pyrimidine-4-substituted-2,6-triamines were obtained in varying yields. They note slower reaction rates and poorer yields for the preparation of the 2,4-diamino compounds than in the preparation of the 2-amino-4-oxo compounds could be that the mildly basic conditions used for the Michael addition step increased the reactivity of the 2-amino-4-oxo pyrimidine
through formation of the anion. Condensation of 119b-123b to 4-substituted-5-aryl-7H-
pyrrolo[2,3-d]pyrimidine-2-amines 124b-128b were accomplished using base.

**Scheme 28.** Synthesis of pyrrolo[2,3-d]pyrimidines from 2-bromo-1,1-diethoxyethane

![Scheme 28](image_url)

El-Gamal and Oh\(^{250}\) synthesized the intermediate 2-(2,2-diethoxyethyl)malononitrile 130b (Scheme 28) from 2-bromo-1,1-diethoxyethane 129b with malononitrile by heating in \(N,N\)-dimethylformamide (DMF) in the presence of base \(K_2CO_3\). Synthesis of 4,6-diamino-5-(2,2-diethoxyethyl)pyrimidine-2-thiolate 131b was carried out by refluxing the geminal dicyano 130b with thiourea in the presence of a stronger base potassium tert-butoxide. Cyclization to 131b could be achieved by neutralization of the thiol potassium salt 130b using 5 N aqueous HCl followed by heating with 10 N aqueous NaOH. Reduction of the thiol compound 131b using Raney nickel afforded 7H-pyrrolo[2,3-d]pyrimidin-4-amine 132b.

Gangjee et al.\(^{251}\) attempted synthesis of 2-amino-pyrrolo[2,3-d]pyrimidines by reacting 3-acetyldihydrofuran-2(3H)-one 133b (Scheme 29) and guanidine carbonate in refluxing conditions with absolute ethanol in the presence of triethylamine to afford 136b. It was converted to 4-chloro-5-(2-chloroethyl)-6-methylpyrimidin-2-amine 135b by refluxing with phosphorus oxychloride. Condensation of benzylamine with 135b in the presence of triethylamine under reflux in n-BuOH afforded the bicyclic 7-benzyl-4-methyl-6,7-dihydro-5H-pyrrolo[2,3-d]pyrimidin-2-amine 136b. Oxidation to 137b was carried out using the
manganese dioxide. Removal of benzyl protecting group was carried out using liquid ammonia to afford the target compound 4-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-amine 138b.

**Scheme 29.** Synthesis of 2-amino-pyrrolo[2,3-d]pyrimidines from 3-acetyldihydrofuran-2(3H)-one

B.5. Pyrrolo[3,2-d]pyrimidines

**Scheme 30.** Synthesis of pyrrolo[3,2-d]pyrimidines from (2-substituted-pyrimidin-5-yl)boronic acids

Beveridge and Gerstenberger reported one-pot four-step cascade sequence to synthesize pyrrolo[3,2-d]pyrimidines via reaction of (2-substituted-pyrimidin-5-yl)boronic acids.
acids 139b with di-tert-butyl-azodicarboxylate (DBAD) and 1,3-dicarbonyls 141b (Scheme 30). The reaction proceeds via a with key steps involving a copper-catalyzed boronic acid coupling to DBAD and a Fischer indolization to provide 2-,6- and 7- substituted pyrrolo[3,2-d]pyrimidines 142b-144b.

Scheme 31. Synthesis of 2-amino 4-oxo pyrrolo[3,2-d]pyrimidines from 2-amino-6-methylpyrimidin-4(3H)-one

\[
\begin{align*}
\text{HN} & \quad \text{HN} \\
\text{H}_2\text{N} & \quad \text{H}_2\text{N} \\
\text{O} & \quad \text{O} \\
\text{145b} & \quad \text{146b} \\
\text{H}_2\text{SO}_4, \text{HNO}_3 & \quad \text{DMF dimethylacetal DMF} \\
\text{147b} & \quad \text{148b}
\end{align*}
\]

36% over three steps

Furneaux et al.253 reported the synthesis of 2-amino-3H,5H-pyrrolo[3,2-d]pyrimidin-4-one 148b (Scheme 31). Nitration of 2-amino-6-methylpyrimidin-4(3H)-one 145b afforded compound 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one, 146b. On treatment of nitro derivative 146b with 6 equivalent of DMF dimethyl acetal in DMF at 100 °C, compound (E)-N’-(4-((E)-2-(dimethylamino)vinyl)-5-nitro-6-oxo-1,6-dihydropyrimidin-2-yl)-N,N-dimethylformimidamide, 147b is obtained which upon subsequent dithionite reduction and cyclization affords pyrrolo[3,2-d]pyrimidine 148b.

Taylor et al.254 obtained (E)-N’-(4-((E)-2-(dimethylamino)vinyl)-1-methyl-5-nitro-6-oxo-1,6-dihydropyrimidin-2-yl)-N,N-dimethylformimidamide, 150b (Scheme 32) upon reaction of 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one, 149b with DMF dimethyl acetal.
Subsequent addition of sodium dithionite provided \((E)-N,N\text{-dimethyl}-N'-(3\text{-methyl-4-oxo-4,5-dihydro-}3H\text{-pyrrolo}[3,2-\text{d}]\text{pyrimidin-2-yl})\text{formimidamide}~151b\), which hydrolysis provides 2-amino-3-methyl-3,5-dihydro-4\text{-}H\text{-pyrrolo}[3,2-\text{d}]\text{pyrimidin-4-one}~152b.

**Scheme 32.** Synthesis of 2-amino 4-oxo pyrrolo[3,2-\text{d}]pyrimidines from 2-amino-6-methyl-5-nitropyrimidin-4(3\text{H})-one

\[
\begin{align*}
149b & \xrightarrow{\text{DMF, dimethylacetel}} 150b & \xrightarrow{\text{Na}_2\text{S}_2\text{O}_4, \text{H}_2\text{O/THF}} 151b \\
1\text{N NaOH} & \xrightarrow{} 152b
\end{align*}
\]

Tian *et al.*\textsuperscript{234} condensed \((Z)-4-(\text{benzyloxy})-3\text{-hydroxybut-2-enenitrile,}~153b\) (Scheme 33) with diethyl aminomalonate in the present of acetic acid to afford intermediate 154b, which was further cyclized under basic condition to produce ethyl 3-amino-5-((benzyloxy)methyl)-1\text{H}\text{-pyrrole-2-carboxylate,}~155b\) using methodology published by Gangjee *et al.*\textsuperscript{255} The pyrrole 155b was condensed with 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea with acetic acid as catalyst in MeOH to afford 156b. The self-condensation reaction of 156b could be processed in the present of sodium methoxide in MeOH to obtain \((6-((\text{benzyloxy})\text{methyl})-4\text{-oxo-4,5-dihydro-}3\text{H}\text{-pyrrolo}[3,2-\text{d}]\text{pyrimidin-2-yl})\text{carbamate,}~157b\). Hydrolysis of the carbamate group with aqueous sodium hydroxide at 60 °C afforded pyrrolo[3,2-\text{d}]pyrimidine 158b.
Scheme 33. Synthesis of 2-amino 4-oxo pyrrolo[3,2-\(d\)]pyrimidines from (Z)-4-(benzyloxy)-3-hydroxybut-2-enenitrile

Scheme 34. Synthesis of 2-alkyl-4-oxo-pyrrolo[3,2-\(d\)]pyrimidines from ethyl 3-amino-5-substituted-1\(H\)-pyrrole-2-carboxylate

Pyrrolo[3,2-\(d\)]pyrimidines with 2-alkyl substituents are obtained by treatment of 5-substituted pyrrole 159b (Scheme 34) with an alkyl nitriles in anhydrous hydrochloric acid followed by heating with sodium hydroxide.\(^{256}\) For R = methyl, the yield was 53%.

Pyrrolo[3,2-\(d\)]pyrimidines with 2-\(H\) are obtained by cyclocondensation of substituted ethyl 3-amino-1\(H\)-pyrrole-2-carboxylate 161b (Scheme 35) with formamidine in refluxing ethanol.\(^{253, 257}\)
Scheme 35. Synthesis of 3H-pyrrolo[3,2-d]pyrimidin-4(5H)-one from ethyl 3-amino-1H-pyrrole-2-carboxylate

Scheme 36. Synthesis of 2-sulfanyl-4-oxo-pyrrolo[3,2-d]pyrimidines from ethyl 3-amino-1H-pyrrole-2-carboxylate

Pyrrolo[3,2-d]pyrimidine 164b 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 163b (Scheme 36) with an ethyl isothiocyanatoformate and hydrochloric acid in benzene at reflux followed by heating with aqueous sodium hydroxide.\textsuperscript{237}

Scheme 37. Synthesis of 2-amino-4-oxo-pyrrolo[3,2-d]pyrimidines from ethyl 3-amino-5-phenyl-1H-pyrrole-2-carboxylate

Pyrrolo[3,2-d]pyrimidine 165b with 2-amino substituents are obtained by treatment of from ethyl 3-amino-5-phenyl-1H-pyrrole-2-carboxylate 163b (Scheme 37) with cyanamide and acid in dioxane at reflux followed by heating with aqueous sodium hydroxide.\textsuperscript{256}
**Scheme 38.** Synthesis of pyrrolo[3,2-\(d\)]pyrimidine 2,4-dione from ethyl 3-amino-5-phenyl-1\(H\)-pyrrole-2-carboxylate

![Scheme 38](attachment:image.png)

Pyrrolo[3,2-\(d\)]pyrimidine 166b with 2,4-dioxo substituent is obtained by cyclocondensation of ethyl 3-amino-5-phenyl-1\(H\)-pyrrole-2-carboxylate 163b (Scheme 38) with potassium cyanate under acidic conditions at room temperature followed by heating with aqueous sodium hydroxide.\(^{258}\)

**Scheme 39.** Synthesis of 5-substituted ethyl 3-amino-1\(H\)-pyrrole-2-carboxylate from substituted cyanoacetylene

![Scheme 39](attachment:image.png)

Treatment of substituted cyanoacetylenes 168b (Scheme 39) with diethylaminomalonate 167b followed by a base catalyzed intramolecular cyclization affords substituted ethyl 3-amino-1\(H\)-pyrrole-2-carboxylate 169b-171b.\(^{259}\) The pyrroles can be cyclized to afford 2-substituted 4-oxo pyrrolo[3,2-\(d\)]pyrimidines, as exemplified in Scheme 33
and 37 (2-amino), Scheme 34 (2-alkyls), Scheme 35 (2-H), Scheme 36 (2-sulfanyl) and Scheme 38 (2-oxo).

**Scheme 40.** Synthesis of 5-substituted ethyl 3-amino-1H-pyrrole-2-carboxylate from 3-oxo-3-substituted propanenitrile

Treatment of 3-oxo-3-phenylpropanenitrile 172b (**Scheme 40**) with p-toluenesulfonic anhydride provides the corresponding 2-cyano-1-phenylvinyl 4-methylbenzenesulfonate 173b. The crude enol ester 173b is immediately condensed with diethyl aminomalonate using sodium ethoxide to provide ethyl 3-amino-5-phenyl-1H-pyrrole-2-carboxylate 165b. Similar to Scheme 36, the pyrrole can be further cyclized to 2-substituted pyrrolo[3,2-\(d\)]pyrimidines.
III. STATEMENT OF THE PROBLEM

The present work deals with the design and molecular modeling studies in the following three major areas:

A. Single agents with combination chemotherapy potential

A.1. Inhibition of multiple tyrosine kinases and microtubule targeting agents:
Quinazoline and Pyrido[3,2-\(d\)]pyrimidine analogs as multiple tyrosine kinase inhibitors and microtubule targeting agents

A.2. Single agents with TS and multiple tyrosine kinase inhibitors:
Pyrimido[4,5-\(b\)]indoles as receptor tyrosine kinase and thymidylate synthase inhibitors

B. Folate Receptors (FR) and/or Proton-Coupled Folate Transporter (PCFT) targeted (cytosolic and/or mitochondrial) one-carbon (1C) metabolism inhibitors

B.1 Inhibitors of de novo purine biosynthetic enzymes (cytosolic 1C metabolism)
1. 5-Substituted, 2-amino-4-oxo pyrrolo[2,3-\(d\)]pyrimidine-pyridyl classical antifolates

B.2 Dual inhibitors of cytosolic (de novo purine biosynthetic) and mitochondrial (serine hydroxymethyltransferase 2) 1C metabolism enzymes
1. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-\(d\)]pyrimidine-pyridyl classical antifolates
2. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-\(d\)]pyrimidine-3’-fluoropyridyl classical antifolates

B.3 5-Substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-\(d\)]/[3,2-\(d\)]pyrimidine-pyridyl classical antifolates

C. Selective inhibition of \(P. jirovecii\) dihydrofolate reductase
1. 5-Substituted, 2,4-diamino pyrimidines (trimethoprim analogs) as selective pjDHFR inhibitors
A. Single agents with combination chemotherapy potential

Angiogenesis – the process of formation of new blood vessels from existing vasculature – is essential for tumor growth and metastasis.\textsuperscript{58} When a tumor grows beyond 2 mm\textsuperscript{3}, it requires nutrients and oxygen for its growth and survival and thus initiates angiogenesis. Under hypoxic conditions, tumors secrete proangiogenic growth factors such as VEGF, PDGF, and EGF, key mediators of angiogenesis. These growth factors bind to their respective RTKs (VEGFR, PDGFR-β, and EGFR) and stimulate the process of angiogenesis, resulting in tumor growth, survival, and metastases. Agents that circumvent angiogenesis by inhibition of RTKs have established a new paradigm in cancer chemotherapy.\textsuperscript{57} 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{261, 262} 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-β, and Raf kinase) and sunitinib (inhibits VEGFRs, PDGFRs, and c-kit).\textsuperscript{263} However, the antiangiogenic treatment only prevents blood supply to the tumor but does not destroy cancer cells. Thus antiangiogenic agents need to be combined with radiotherapy or chemotherapy to form an effective therapy and achieve synergistic effects.\textsuperscript{264}

Inhibitors of TS and tubulin are widely used cytotoxic agents in cancer chemotherapy. While inhibition of the enzyme TS prevents the \textit{de novo} synthesis of nucleotides crucial for DNA synthesis,\textsuperscript{41} tubulin inhibitors target microtubule dynamics involved in mitosis.\textsuperscript{5}

TS catalyzes the \textit{de novo} synthesis of dTMP from dUMP utilizing 5,10-CH\textsubscript{2}THF as the cofactor.\textsuperscript{40} Because of its vital role in DNA synthesis and cell growth, TS is a viable target for several clinically used cancer chemotherapeutic agents.\textsuperscript{41} Nucleoside–based inhibitors such as 5-FU and capecitabine have found extensive utility in ovarian, breast, colon, and several other
cancers alone and in combinations and are a mainstay in cancer chemotherapy.\textsuperscript{44,46} Antifolates, including raltitrexed and pemetrexed, are TS inhibitors clinically used, alone or in combination, in the treatment of mesotheliomas, non-small cell lung, and advanced colorectal cancers.\textsuperscript{49,265}

Since microtubule dynamics plays a crucial role in mitosis and cell division,\textsuperscript{5} microtubule targeting agents represent an important class of anticancer agents. Tubulin binding agents such as paclitaxel and vinca alkaloids are widely used to treat solid tumors and hematological malignancies.\textsuperscript{263,266,267}

Combination chemotherapy with antiangiogenic agents and cytotoxic agents is more effective in cancer treatment than either agent alone.\textsuperscript{264,268} Single agents with dual antiangiogenic and cytotoxic activities significantly decreased tumor growth, tumor metastasis and angiogenesis superior to docetaxel and sunitinib in xenograft mice models, remarkably without any toxicity.\textsuperscript{122,123} Such single agents with multiple mechanisms of action are commonly referred to as designed multiple ligands and have several pharmacokinetic and pharmacodynamic advantages over separately administered agents. These agents could potentially avoid drug-drug interactions and pharmacokinetic problems associated with two or more agents.\textsuperscript{269,270} Also, they could prevent or delay the emergence of resistance and not cause overlapping toxicities.\textsuperscript{269,271} Most significantly, single agents with dual cytotoxic and antiangiogenic activities simultaneously target rapidly proliferating tumor cells and the tumor vasculature. Also, such single agents could afford synergistic effects as they can exert their cytotoxic effect as soon as and during transient tumor vasculature normalization caused by the antiangiogenic component.\textsuperscript{272} As a result, structural design of such multitargeted single agents should allow the cytotoxicity to be manifested as soon as the antiangiogenic effects are operable. 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{273} 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{274-277} The single agents offer other advantages such as decreased cost and increased patient compliance,\textsuperscript{270} which can play a major part in the clinical success of therapy.

The antiangiogenic component of single agents targets RTK-overexpressing endothelial cells and as a result, is typically targeted to tumor cells under normal circumstances.\textsuperscript{58} In contrast, 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{271} Therefore, a key challenge in the design of single agents with a cytotoxic component is that the cytotoxic component should only destroy tumor cells that are compromised \textit{via} the antiangiogenic effect but should not cause toxicity to normal cells not affected by the antiangiogenic effect.\textsuperscript{123} As a result, the cytotoxic component of these single agents need not be as potent as conventional chemotherapeutic agents and hence should avoid dose-limiting toxicities\textsuperscript{278} associated with other cytotoxic agents.

Single agents with dual antiangiogenic and cytotoxic activities significantly decreased tumor growth, tumor metastasis and angiogenesis in xenograft mice models, remarkably without any toxicity.\textsuperscript{118, 122, 123, 279} The antiangiogenic effects of these compounds were due to inhibition of RTKs and the cytotoxic effects were due to inhibition of thymidylate synthase\textsuperscript{123} or tubulin\textsuperscript{118, 122} or dihydrofolate reductase.\textsuperscript{279} In these studies, VEGFR-2, PDGFR-\(\beta\), and EGFR were the chosen targets for antiangiogenic effects because of their crucial role in angiogenesis. Additionally, the successful clinical and preclinical combinations of TS inhibitors (\textit{e.g.}, capecitabine, pemetrexed) or tubulin inhibitors like paclitaxel with antiangiogenic agents was also an important factor in selecting TS or tubulin as the potential cytotoxic targets.\textsuperscript{260-284}
A.1. Inhibition of multiple tyrosine kinases and microtubule targeting agents

A.1.1. Design of quinazoline and pyrido[3,2-d]pyrimidine analogs as multiple tyrosine kinase inhibitors and microtubule targeting agents

![Chemical structures](image)

**Figure 39.** Structure of the lead compounds.

Compound 33c (**Fig. 39**) was reported as a potent inducer of apoptosis, binding at the colchicine site and an inhibitor of tubulin polymerization (EC$_{50}$ T47D = 2 ± 0.1 nM). The compound was also shown to overcome resistance due to multidrug-resistant transporters such as P-glycoprotein (Pgp), breast cancer resistance protein (BCRP1) and was able to cross the BBB to attain 30-fold higher concentration in the brain compared to the plasma. These pharmacokinetic parameters led to the evaluation of 33c in glioblastoma multiforme (GBM)
and other cancers, and it is currently approved by FDA as an orphan drug in the treatment of GBM.\textsuperscript{286} Derivatives of 33c (34c-42c, Fig. 39) with different substitutions at the 2-position (CH$_3$, Cl or H) and 4-position (anilines) were synthesized by Choudhary and Gangjee.\textsuperscript{117}

**Table 4.** Inhibition of microtubule polymerization, antiproliferative activity and cellular inhibition of VEGFR-2 and EGFR

<table>
<thead>
<tr>
<th>Compound</th>
<th>Microtubule depolymerization EC$_{50}$ (nM)</th>
<th>Antiproliferative effects</th>
<th>Cellular RTK Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA-MB-435 IC$_{50}$ (nM) ± SD</td>
<td>VEGFR2 IC$_{50}$ (nM) ± SD</td>
<td>EGFR IC$_{50}$ (nM) ± SD</td>
</tr>
<tr>
<td>33c</td>
<td>2.1</td>
<td>1.7 ± 0.1</td>
<td>8.4 ± 2.2</td>
</tr>
<tr>
<td>34c</td>
<td>3</td>
<td>1.1 ± 0.2</td>
<td>9.3 ± 3.9</td>
</tr>
<tr>
<td>35c</td>
<td>2</td>
<td>1.2 ± 0.2</td>
<td>14.7 ± 2.8</td>
</tr>
<tr>
<td>36c</td>
<td>2.4</td>
<td>2.0 ± 0.4</td>
<td>16.8 ± 4.1</td>
</tr>
<tr>
<td>37c</td>
<td>2</td>
<td>0.6 ± 0.1</td>
<td>97.5 ± 10.1</td>
</tr>
<tr>
<td>38c</td>
<td>2</td>
<td>0.7 ± 0.2</td>
<td>55.2 ± 11.1</td>
</tr>
<tr>
<td>39c</td>
<td>1.9</td>
<td>1.4 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>40c</td>
<td>2.1</td>
<td>7.1 ± 0.8</td>
<td>ND</td>
</tr>
<tr>
<td>41c</td>
<td>3</td>
<td>12.4 ± 1.7</td>
<td>111.1 ± 19.2</td>
</tr>
<tr>
<td>42c</td>
<td>2</td>
<td>7.8 ± 0.8</td>
<td>ND</td>
</tr>
<tr>
<td>CA4</td>
<td>9.8</td>
<td>4.4 ± 0.46</td>
<td>ND</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>ND</td>
<td>ND</td>
<td>18.9 ± 2.7</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>ND</td>
<td>ND</td>
<td>124.7 ± 18.2</td>
</tr>
</tbody>
</table>

ND- Not Determined

In the antiproliferative assays and the microtubule depolymerization assays compounds 33c-42c displayed potent inhibitory activities (Table 4). The 2-H substituted compounds (40c-42c) were 7- to 10-fold less potent than the 2-Me (33c-36c) and 2-Cl (37c-39c) substituted compounds, suggesting bulk is important for antitubulin activity at the 2-position of the quinazoline scaffold. In the RTK inhibitory assays, the 2-Me and 2-Cl substituted compounds
were also more potent than 2-H substituted compounds. For the 2-substituted quinazolines (33c-36c), varying the aniline substitution at 4-position was well tolerated. The 2-Cl substituted compound 37c was a potent inhibitor of EGFR but resulted in the loss of 10-fold and 15-fold activity in VEGFR-2 and PDGFR-β, respectively when compared to corresponding 2-Me compound 33c.

Table 5. IC<sub>50</sub>s in parent cell lines (HeLa and SKOV3), βIII- and Pgp-overexpressing resistant cancer cell lines (WTβ3 and M6/6, respectively)

<table>
<thead>
<tr>
<th>#</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD in HeLa (nM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD in WTβ3 (nM)</th>
<th>Rr Value (WT/HeLa)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD in SKOV3 Cells (nM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD in M6/6 Cells (nM)</th>
<th>Rr Value (M6/6/SKOV3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33c</td>
<td>0.6 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.0</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>1.4</td>
</tr>
<tr>
<td>34c</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>1.2</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>35c</td>
<td>2.8 ± 0.5</td>
<td>1.6 ± 0.3</td>
<td>0.6</td>
<td>2.6 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>36c</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.8</td>
<td>2.2 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>37c</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.4</td>
<td>1.0</td>
<td>1.5 ± 0.3</td>
<td>2.2 ± 0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>38c</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>0.8</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>39c</td>
<td>2.5 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>0.7</td>
<td>2.9 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>40c</td>
<td>8.6 ± 0.3</td>
<td>7.4 ± 0.7</td>
<td>0.9</td>
<td>10.4 ± 1.1</td>
<td>14.3 ± 0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>41c</td>
<td>16.8 ± 2.2</td>
<td>13.1 ± 0.4</td>
<td>0.8</td>
<td>18.1 ± 1.8</td>
<td>23.4 ± 4.5</td>
<td>1.3</td>
</tr>
<tr>
<td>42c</td>
<td>8.5 ± 0.8</td>
<td>7.3 ± 0.4</td>
<td>0.6</td>
<td>13.6 ± 0.9</td>
<td>15.6 ± 1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>CA-4</td>
<td>3 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>1</td>
<td>5.5 ± 0.5</td>
<td>7.2 ± 1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.8 ± 0.36</td>
<td>24 ± 3</td>
<td>8.6</td>
<td>5 ± 0.6</td>
<td>1200 ± 58</td>
<td>240</td>
</tr>
</tbody>
</table>

The lead compounds were also tested against Pgp- and βIII-overexpressing cell lines to determine their efficacy against the most common type of resistance that renders current MTAs such as the vinca alkaloids and taxanes ineffective. The colchicine site agents like
CA-4 and the lead compounds 33c-42c circumvent these resistance mechanisms as evidenced by relative resistance (Rr) values close to 1 or lower than 1 (Table 5). However, paclitaxel is ineffective against these resistant cell line with Rr values ∼9 and 240 for βIII- and Pgp-overexpressing resistant cancer cell lines (WTβ3 and M6/6), respectively. These data suggest that these compounds overcome both Pgp and βIII-tubulin mediated resistance and in that regard, have advantages over paclitaxel.

![Chemical Structures]

**Figure 40.** Few of the representative 6,6-bicyclic based RTK inhibitors.

The quinazoline scaffold has also been widely explored for its RTK inhibitory activity as exemplified by gefitinib (EGFR), afatinib (EGFR), erlotinib (EGFR) and vandetanib (VEGFR-2, VEGFR-3, and EGFR) (Fig. 40). Structure-activity relationships of the quinazoline scaffold, to date, include only N4-desmethyl analogs as EGFR inhibitors (Fig. 40) which suggested that the quinazoline scaffold showed bulk tolerance at the 6- and 7-positions. However, the corresponding N4-Me analogs were not explored for their RTK inhibitory activities. The N4-Me analogs presented above (Fig. 39) not only showed potent
antitubulin activity but also inhibited one or more RTKs in the nanomolar range, suggesting that 2,4-disubstituted quinazoline analogs act as single agents with a combination of tubulin depolymerization and antiangiogenic activity.

![Structure of proposed compounds in Series I.](image)

**Figure 41.** Structure of proposed compounds in Series I.

Compounds 1c and 2c (Series I) are designed to vary substitutions at 2- and 4-positions of the quinazoline scaffold. The 2-Cl substitution in the lead compounds showed the most potent microtubule depolymerization activity compared to the 2-CH₃ and 2-H substitutions and also showed potent EGFR inhibition (IC₅₀ = 3.6 to 10.6 nM) with moderate VEGFR-2 and PDGFR-β inhibition. The 4’-SCH₃ substitution on the Nᵗ-aryl group also produced potent antitubulin activity (Table 4). Therefore, compound 1c combining these two pharmacophores is predicted to be a single agent with potent microtubule depolymerization and RTK inhibitory activity. The 2-H substitution of compound 2c mimics the RTK inhibitors, which will allow hinge region binding and maintain interaction with the colchicine binding site in tubulin.

We performed molecular docking studies using Maestro 11.9 on the proposed compounds 1c and 2c, and one of the most active leads compound 5 in tubulin (PDB: 4O2B), VEGFR-2 (PDB: 4AG8) and EGFR (PDB: 1M17) to determine their binding poses and ligand-interactions. The molecular modeling protocol was validated by redocking the crystallized ligands colchicine, axitinib and erlotinib in tubulin, VEGFR-2, and EGFR, respectively with RMSD of 0.17 Å, 0.50 Å, and 1.19 Å, respectively.
Figure 42. A) Superimposition of the docked poses of 37c (orange) and 1c (pink) and B) docked pose of 2c (cyan) in the colchicine binding site of tubulin (PDB ID: 4O2B).290

The docked poses of 1c (Fig. 42A) and 2c (Fig. 42B) are very similar to the docked pose of the lead compound 37c in the colchicine site of the tubulin. The N1 makes water-mediated H-bond with the Cys241. The quinazoline scaffold sits deep in the β-tubulin pocket lined by hydrophobic residues such as Ile318, Ala354, Ile378, Ala316, Ala250, Leu252, Leu242, Val238, and Leu255. The 2-position substitution orients toward the pocket lined by Ala250, Leu242, and Leu252, the 2-Cl substitution makes favorable hydrophobic interaction with the pocket, whereas the 2-H group does not interact with the pocket. This results in a better docking score for the 2-Cl compound 1c compared to the 2-H compound 2c. Due to a similar reason, the lead compounds with the 2-Cl and 2-CH₃ substitutions were more active than the 2-H lead compounds. These 2-position variations can allow for optimization of the antitubulin activity and hence the cytotoxic activity of these potential single agents. The N⁴-Me group makes hydrophobic interaction with Ala250 whereas the N⁴-aryl ring orients toward the pocket lined by the β-tubulin residues, Ala316, Met259, Asn258, Lys352, Thr317 and the α-tubulin residues Ala180 and Val181 residues. The 4'-SMe group of compound 1c is more
hydrophobic in comparison to the 4’-OMe group of the lead 37c and can make better hydrophobic interaction with hydrophobic residues of α-tubulin. This results in a better docking score for 1c (-9.60 kcal/mol) than 37c (-9.08 kcal/mol). The 4’-OMe-phenyl and 4’-SMe-phenyl rings of 37c and 1c do not fully occupy the pocket at the α- and β-tubulin interface, whereas the docked pose of 2c shows that the bulky 2-naphthyl ring at the N^4-position goes deeper in the pocket. Such a bulkier group at the interface of the heterodimer should produce the more curved structure of the microtubule, thus further reducing polymerization.\(^1\)

Compounds 1c (-9.60 kcal/mol) and 2c (-9.88 kcal/mol) have better docking scores than 37c (-9.08 kcal/mol), suggesting that these compounds should have similar or better activity than 37c as MTAs binding to the colchicine site.

**Figure 43.** A) Superimposition of the docked pose of 1c (pink) and crystallized ligand axitinib (green) and B) docked pose of 2c (cyan) in the axitinib binding site of VEGFR-2 (PDB ID: 4AG8).\(^{291}\)

The docked poses of 1c and 2c in the binding site of VEGFR-2 (Fig. 43) show that the quinazoline scaffold binds to the pocket where the thiobenzamide of axitinib binds and
interacts with residues Lys868 (cation-pi interaction), Val899 and Val914. The 4-position aniline substituents interact with Val848, Ala866, Leu1035 and Phe1047 (face-to-edge pi-pi stacking interaction) and bind in the pocket where the indazole scaffold of axitinib binds. The docking scores of 1c and 2c in VEGFR-2 were -10.86 and -12.00 kcal/mol, respectively, compared to axitinib which had a docking score of -12.44 kcal/mol.

**Figure 44.** A) Superimposition of the crystallized ligand Erlotinib (orange) and 1c (pink) and B) docked pose of 2c (cyan) in the erlotinib binding site of EGFR (PDB: 1M17).

**Fig. 44A** shows the superimposition of the docked pose of 1c with the crystallized ligand erlotinib in the EGFR binding site. The quinazoline scaffolds of both ligands overlap well with each other, N1 H-bonds with the Met769 backbone NH, N3 makes a water-mediated H-bond with Thr766 and a dipole-dipole interaction with the backbone CO of Met769. The 2-Cl group of 1c makes a halogen bond with the backbone CO of Gln767 in addition to the NH with hinge region (pink colored dotted line shown in Fig. 44A). The N\(^4\)-aryl groups of erlotinib and 1c occupy the hydrophobic pocket of EGFR near the DFG motif in the kinase binding site and the hydrophobic 4'-SMe group occupies the same region as the acetylene group of the erlotinib. In comparison, the 2-H compound 2c makes H-bond interactions with the hinge region like erlotinib, where 2-H makes dipole-dipole interaction with the backbone CO of the
Gln767. The 2-naphthyl group of 2c binds in the hydrophobic pocket and also interacts with the Asp831 of the DFG motif and the OMe group H-bonds with Thr830. Overall, the proposed compounds in Series I make unique interactions with the EGFR binding site while maintaining the interactions of erlotinib, the standard EGFR inhibitor. The docking scores of 1c and 2c were -8.21 and -9.09 kcal/mol, respectively compared to erlotinib, which had a docking score of -9.29 kcal/mol. Overall, based on our in-silico studies and good docking scores in the tubulin, EGFR and VEGFR-2 binding sites we predict that the compounds 1c and 2c (Series I) should have potent inhibitory activity at all three targets.

Figure 45. Superimposition of the docked poses of 1c (left) and 2c (right) in the binding sites of EGFR, VEGFR-2, and tubulin.

To determine the change in conformations these molecules undergo to bind to the three separate targets, the docked conformations of 1c and 2c in all three targets were superimposed (Fig. 45). The conformations of these molecules primarily differ in the \(N^4\)-Me- and anilino orientations. The \(N^4\)-aryl moieties of both 1c and 2c in the tubulin and VEGFR-2 binding sites orient toward the 5-position of the quinazoline scaffolds, whereas in the EGFR binding site the \(N^4\)-Me group orients toward the 5-position. Therefore, rotation around the C4-N4 bond is necessary to adopt these opposite conformations to bind to multiple targets, which are restricted due to a steric clash between the 5-CH and the methyl or aryl groups depicted in Fig 45.
Verubulin and its derivatives have been effective MTAs and VDAs but still suffer from narrow therapeutic indices causing cardiotoxicity, thus have not been successful in clinical trials so far. The benzene rings of the quinazoline scaffold of compounds 33c-42c, and 1c-2c were predicted, by Maestro, to be liable for metabolic oxidation by CYP450.

**Figure 46.** Structure of proposed compounds in Series II.

Introduction of a nitrogen atom is a high-impact design element for multiparameter optimization such as improvement of the therapeutic index and/or metabolic liability of small molecules. Therefore, we propose compounds 3c-10c (Series II, Fig. 46) with a pyrido[3,2-d]pyrimidine scaffold in place of the quinazoline.

**Figure 47.** Rotational barrier around the C4-N4 bond (kcal/mol) for A) quinazoline analogs (37c-42c and 1c-2c), and B) pyrido[3,2-d]pyrimidine analogs (3c-10c), prediction with Maestro 11.9.

Introduction of the nitrogen atom in the scaffold can influence the conformation of these compounds. The quinazolines like 1c and 2c (Fig. 41) need the flexibility to rotate around
the C4-N4 bond to bind to multiple targets which is hindered due to the steric clash of either \(N^1\)-aryl or \(N^4\)-methyl moiety with the hydrogen of 5-CH, thus replacing the 5-CH group with a \(sp^2\) nitrogen atom is hypothesized to reduce the steric clash and thereby decrease the rotational barrier around C4-N4 bond. Using molecular modeling, the rotational barrier around the C4-N4 bond of both the quinazoline compounds and corresponding pyrido[3,2-\(d\)]pyrimidine compounds were determined (Fig. 47). This was done by carrying out a coordinate (dihedral) scan around the bond and plotting the relative energies of the molecules versus the dihedral angle. The plot clearly shows that the quinazoline compounds (Fig. 47) have higher rotational barriers (-7.5 to -15 kcal/mol) compared to the pyrido[3,2-\(d\)]pyrimidines (Series II, -3 to -4 kcal/mol), indicating that compounds in Series II (Fig. 46) should afford greater conformational flexibility than the lead quinazoline compounds. This should allow these compounds to adopt different conformations necessary to bind the multiple targets (tubulin, EGFR, and VEGFR-2), more easily than the quinazoline analogs (37c-42c and 1c-2c) and thus increase potency.

![Figure 48](image)

**Figure 48.** Cytochrome P450 metabolism prediction showing metabolically liable sites (green circles) on A) pyrido[3,2-\(d\)]pyrimidine analog (6c) and B) quinazoline analog (39c), Maestro 11.9.\(^{289}\)

Maestro also predicted that replacement of 5-CH of the quinazoline with a N atom removes the metabolic liability of the benzene ring of the quinazoline (Fig. 48). Thus, such
small change can have several important effects on molecular and physicochemical properties and hence enhance clinical utility.

Figure 49. A) Docked poses of 39c (cyan) and B) 6c (pink) in the colchicine binding site of tubulin (PDB ID: 4O2B), orange dashed line depicts steric clash within the ligand.

Compounds in Series II were docked in the colchicine binding site of tubulin. Fig. 49 shows the docked poses of the quinazoline analog 39c and the pyrido[3,2-d]pyrimidine analog 6c in the binding site of tubulin. Both ligands show similar interactions with the tubulin binding site. However, the pyridine containing compound 6c (-10.21 kcal/mol) shows a better docking score than the lead 39c (-8.33 kcal/mol). This is, partially, attributed to the relief of intra-ligand strain caused by a steric clash (orange dashed line, Fig. 49A) between the naphthalene ring and the 5-CH hydrogen in the docked pose of 39c, which is absent in 6c. Similarly, due to a lock of the steric clash other compounds in the Series II (-8.66 to -10.46 kcal/mol) also showed better docking scores than the corresponding quinazoline analogs (-8.07 to -9.39 kcal/mol) because of the same reason. Compounds 3c-10c showed similar binding and good docking scores compared to the lead compounds in both EGFR and VEGFR-2. With potentially decreased metabolic liability and increased docking scores, conformational flexibility and
water solubility induced by the replacement of the aromatic CH with a nitrogen atom provide a strong rationale for the synthesis of compounds in Series II.

A.2. Single agents with TS and multiple tyrosine kinase inhibitors: Pyrimido[4,5-b]indoles as receptor tyrosine kinase and thymidylate synthase inhibitors

Gangjee et al.\textsuperscript{123} reported tricyclic pyrimido[4,5-b]indoles 43c and 44c (Fig. 50) as dual inhibitors of thymidylate synthase and multiple RTKs (VEGFR-2 and PDGFR\(\beta\)). In COLO-205 mice xenografts, compounds 43c and 44c significantly inhibited tumor growth, tumor vasculature and liver metastasis better than docetaxel and remarkably with no toxicity.

![Figure 50. Structure of lead and proposed compounds in Series III](image)

Table 6. Inhibition of TS, RTKs and angiogenesis by 43c and 44c.\textsuperscript{187}

<table>
<thead>
<tr>
<th></th>
<th>hTS (µM)</th>
<th>EGFR (nM)</th>
<th>VEGFR-2 (nM)</th>
<th>PDGFR-(\beta) (nM)</th>
<th>CAM Angiogenesis (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43c</td>
<td>0.54</td>
<td>15.1 ± 2.5</td>
<td>22.6 ± 2.7</td>
<td>2.8 ± 0.3</td>
<td>28.2 ± 2.9</td>
</tr>
<tr>
<td>44c</td>
<td>0.39</td>
<td>10.41 ± 1.9</td>
<td>56.3 ± 8.2</td>
<td>40.3 ± 6.7</td>
<td>20.3 ± 5.2</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.38</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>ND</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
<td>1.3 ± 0.07</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>ND</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
<td>ND</td>
<td>29.1 ± 1.9</td>
</tr>
</tbody>
</table>

ND- Not Determined

Table 6 shows inhibitory data for 43c and 44c against human TS (hTS) and kinases EGFR, VEGFR-2, and PDGFR-\(\beta\).\textsuperscript{289}
Compounds 11c and 12c (Fig. 50) are analogs of 43c and 44c, respectively, with an oxygen atom at the 5-position in place of a sulfur atom. From molecular modeling of the lead compounds, it was observed that the 5-position phenyl ring of 43c and 44c is surrounded by hydrophobic amino acid residues in VEGFR-2, EGFR, PDGFR-β, and hTS binding sites (Fig. 51). Thus, compounds with an oxygen atom linkers in place of the sulfur atom linker were designed. This modification results in changes in the dihedral angle and bond lengths of the linker region and could allow better interactions with hydrophobic residues in the binding site of the targeted kinases and hTS.

**Figure 51.** Docked poses of A) 43c (cyan) and 44c (peach) in VEGFR-2 (PDB: 4AG8) and B) 11c (green) and 12c (yellow) in VEGFR-2, C) 43c (cyan) in the hTS (PDB: 1JU6) and D) 11c (pink) and 12c (orange) in the hTS binding site.
Compounds 11c and 12c (Fig. 51B) show similar interactions at the VEGFR-2 binding site as the lead compounds 43c and 44c (Fig. 51A). The hinge region binding of the 4-NH$_2$ group of 11c and 12c with Glu917 (C = O); N3 with Cys919 (N-H); 2NH$_2$ with Cys917 (C = O) are observed. The 5-phenoxy rings fit in the hydrophobic pocket with Val899, Leu889, Val899, and Ala866. Thus, the possible inhibition of VEGFR-2 by 11c and 12c is possible. In the hTS crystal structure the C- ring of compounds 43c and 44c (Fig. 51D) show hydrophobic interactions with Trp109, the 5-phenoxy rings make hydrophobic interactions with Ile108, Leu221, and Phe225 and stacking of 11c and 12c with dUMP (pink color) in the binding site is observed (Fig. 51D). Compounds 11c and 12c also showed similar interactions (not shown) with targeted kinases and hTS with docking scores comparable to the lead compounds 43c and 44c (Compound 43c: hTS; -11.39 kcal/mol, VEGFR-2; -10.48 kcal/mol, EGFR; -5.47 kcal/mol and PDGFR-β; -8.47 kcal/mol, Compound 44c: hTS; -11.99 kcal/mol, VEGFR-2; -10.87 kcal/mol, EGFR; -5.61 kcal/mol and PDGFR-β; -8.54 kcal/mol, Compound 11c: hTS; -11.48 kcal/mol, VEGFR-2; -11.63 kcal/mol, EGFR; -6.63 kcal/mol and PDGFR-β; -8.78 kcal/mol, and Compound 12c: hTS; -12.07 kcal/mol, VEGFR-2; -10.38 kcal/mol, EGFR; -6.52 kcal/mol and PDGFR-β; -9.45 kcal/mol). Therefore, these compounds are predicted to be potential single agents with multiple RTKs and hTS inhibitory activities.

B. Folate Receptors (FR) and/or Proton-Coupled Folate Transporter (PCFT) targeted one carbon (1C) metabolism (cytosolic and/or mitochondrial) inhibitors

RFC and PCFT are facilitative folate transporters, whereas FRs (Folate receptors) mediate uptake of folates into cells by receptor-mediated endocytosis. 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. Importantly, FRs in normal tissues are either inaccessible to circulating folates (e.g., FRα in renal tubules) or are nonfunctional (e.g., FRβ in the thymus). 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 acidic pH (pH < 7, optimum at pH 5–5.5), thus limiting PCFT mediated folate transport in most normal tissues at physiological pH (7 – 7.4). 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 of acidic solid tumors and/or FRs is an attractive approach for the development of 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 myeloid lineage including tumor-associated macrophages (TAMs). Based on the pattern 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 completed phase I clinical trials. In addition small molecule N-[4-[2-propyn-1-yl[(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid (ONX0801) has substrate selectivity for FRs over RFC and has TS as its intracellular target. In comparison to the conjugates developed, it is of interest to design single agents with tumor transporter specificity and cytotoxic abilities within a single molecule, which should not require an intracellular cleavage mechanism to release the cytotoxic component. Premature cleavage (before reaching the tumor) of the cytotoxic vinca alkaloids or tubulysin results in unacceptable toxicities.
B.1 Inhibitors of de novo purine biosynthetic enzymes (cytosolic 1C metabolism)

B.1.1. Design of 5-substituted, 2-amino-4-oxo pyrrolo[2,3-d]pyrimidine-pyridyl classical antifolates

Gangjee and coworkers\textsuperscript{185} synthesized 5-substituted pyrrolo[2,3-d]pyrimidine agents (Fig. 52) as dual-acting inhibitors of glycinamide ribonucleotide formyl transferase (GARFTase) and 5-aminoimidazole-4-carboxamide ribonucleotide formyl transferase (AICARFTase) in de novo purine nucleotide biosynthesis (Table 7 and 8). These compounds were substrates for FRs, PCFT, and RFC. Thus, they did not provide tumor-specificity. Like PMX, these analogs (45c-47c) have significant uptake through RFC, which results in dose-limiting toxicity (Table 7) and tumor resistance.

Figure 52. Structures of lead 5-substituted Pyrrolo[2,3-d]pyrimidine analogs

Table 7. IC\textsubscript{50} Values (nM) for 5-substituted Pyrrolo[2,3-d]pyrimidine classical antifolates in RFC-, PCFT-, and FR-expressing CHO cell lines.\textsuperscript{159, 185}
Table 8. Growth inhibition assays: IC$_{50}$ Values (nM) for 5-Substituted Pyrrolo[2,3-$d$]pyrimidines in KB human tumor sublines (expressing RFC, FRα, and PCFT) and protection study results by added metabolites.$^{159, 185}$

<table>
<thead>
<tr>
<th></th>
<th>KB (nM)</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td>PMX</td>
<td>68 ± 12</td>
<td>TS</td>
</tr>
<tr>
<td>45c</td>
<td>68.8 ± 21.2</td>
<td>AICARFTase/GARFTase</td>
</tr>
<tr>
<td>46c</td>
<td>56.6 ± 5.8</td>
<td>AICARFTase/GARFTase</td>
</tr>
<tr>
<td>47c</td>
<td>196.4 ± 55.0</td>
<td>GARFTase</td>
</tr>
</tbody>
</table>

The 5-substituted pyrrolo[2,3-$d$]pyrimidines 45c and 46c, can circumvent the TS inhibition resistance, as these analogs were inhibitors of GARFTase and AICARFTase (Table 8). Dual inhibition in the purine synthesis pathway is beneficial as the tumor cell cannot survive with resistance to only one of the targets. Efforts have been focused on developing agents to achieve: (i) transporter specificity; (ii) dual inhibition of GARFTase and AICARFTase; and (iii) increased KB tumor cell inhibition.

![Figure 53. Structure of proposed compounds in Series IV](image)

The phenyl side-chain, in previously reported$^{185}$ 5-Substituted pyrrolo[2,3-$d$]pyrimidine analogs (Fig. 52), is replaced by a pyridyl side-chain in designed compounds 13c-16c (Series IV, Fig. 53) to induce conformational restriction by promoting an intra-
molecular H-bond between the pyridine nitrogen and the amide NH. The conformational restriction has been successfully used in the drug discovery process to induce selectivity. Thus, a high impact design element such as the pyridyl side-chain to induce conformational restriction is to be studied regarding its potential to induce FRα/PCFT selectivity over RFC by either increasing transport by FRα or PCFT or by decreasing the uptake by RFC. The length of the linker between the pyrrolo[2,3-d]pyrimidine scaffold and the pyridyl glutamate side-chain are varied in the compounds 13c-16c to vary the distance between the two and allow optimization of their activity at multiple targets.

Figure 54. Molecular and physicochemical changes induced by pyridine ring (modified form ref. 295).

Along with the H-bond acceptor capability, changing the benzene ring to the pyridyl ring has multiple effects such as the introduction of a dipole moment, increased polar surface area, decreased lipophilicity, increased water solubility, uneven distribution of electron density, and stronger π-π stacking interactions. Thus, pyridine for benzene substitution can change molecular and physicochemical properties which can influence various intra- and intermolecular orbital, steric, electrostatic and hydrophobic interactions such as dipole-dipole, H-bonding, van der Waals and π-π stacking interactions which can change the pharmacological profile of these compounds.
To understand the binding of these compounds in transporters and intracellular targets, molecular modeling studies of **PMX**, **45c-47c** and **13c-16c** were carried out in the crystal structures of FRs, GARFTase and AICARFTase using Maestro 11.9. As a representative, docking studies of the four-carbon linker lead (**46c**) and corresponding target compound **15c** are presented below.

**Fig. 55** depicts the binding mode of **46c** and **15c** in the active site of FRα. Compounds **46c** and **15c** show similar binding interactions; the 2-NH$_2$ group makes H-bond with the Asp81, the 7-NH makes H-bind with Thr82, the 3-NH makes H-bond with the Ser174, and the 4-oxo makes ion-dipole interaction with the Arg103. The pyrrolo[2,3-$d$]pyrimidine scaffold π-π stacks with Tyr85 and Trp171. The phenyl and pyridyl rings of compounds **46c** and **15c** can make π-π stacking interactions with Trp102, and the electron-deficient pyridyl ring can make a stronger π-π stacking interaction than the phenyl ring with the electron-rich Trp102. The pyridyl nitrogen in **15c** (docking score: -15.49 kcal/mol) is oriented on the same side as the amide NH in the binding site due to the intramolecular H-bond, which resembles the minimum energy pose of compound **15c**. This intramolecular H-bond can provide entropic benefit for the pyridyl analogs (**13c-16c**) in comparison to the lead compounds. The α-COOH group makes an ionic salt bridge with Lys136 and an ion-dipole interaction with Trp138, whereas the γ-COOH group makes ion-dipole interactions with Gln100 and Ser101. The compounds in the Series IV showed similar or better docking scores (-14.75 to -15.49 kcal/mol) than the lead compounds **PMX** and **45c-47c** (-14.74 to -15.11 kcal/mol) suggesting these compounds would be transported by FRα.
Figure 55. A) Docked pose of 46c (cyan) and B) 15c (orange) in the FRα binding site (PDB ID: 5IZQ).

Figure 56. A) Docked pose of 46c (cyan) and B) 15c (orange) in the FRβ binding site (PDB ID: 4KN2).

Fig. 56 shows the docked poses of 46c (-16.03 kcal/mol) and 15c (-16.23 kcal/mol) in the binding site of FRβ. The 2-NH₂ and N1 for both compounds make ion-dipole interactions with the Asp97 side-chain and the 4-oxo groups provide water-mediated H-bonds with Ser190, Arg122 and Arg119 and also H-bonds with His151. The pyridyl ring CH group of 15c makes dipole-dipole interaction with the carbonyl oxygen atom of the backbone of His151. The
docking scores of the compounds 13c-16c suggest that they would display transport through FRβ.

**Figure 57.** A) Docked poses of 46c (cyan) and B) 15c (pink) in the GARFTase binding site (PDB ID: 4ZZ1), substrate GAR showed in orange.

The molecular modeling studies of 46c and 15c in the GARFTase binding site are shown in **Fig. 57**. The pyrrolo[2,3-d]pyrimidine scaffold of these compounds make H-bond interactions with the backbone of Leu899, Asp949, and Ala947. The aromatic side-chains phenyl (46c) and pyridyl (15c) occupy the solvent-exposed binding pocket. Therefore, heteroaromatic side-chains such as the pyridyl ring can make polar interactions with the water molecules in contrast to the hydrophobic phenyl ring of 46c. This should afford better binding of the pyridyl analogs (13c-16c) to GARFTase than the corresponding phenyl analogs (45c-47c). The α-COOH group makes salt bridges with Arg871 and Arg897, whereas the γ-COOH group makes a salt bridge with Lys844 and is solvated by water molecules (**Fig. 57**). The docking scores for 46c and 15c in GARFTase were -13.25 kcal/mol and -14.41 kcal/mol, respectively.
Figure 58. A) Docked poses of 46c (grey) and B) 15c (cyan) in the AICARFTase binding site (PDB ID: 1P4R), substrate AICAR shown in orange.

Compound 46c is a bona fide AICARFTase inhibitor; thus, to compare the binding pose of the compounds in Series IV, we performed molecular modeling studies were carried out in the AICARFTase binding site. Similar to 46c, 15c also showed H-bonding with Asp546, Asn547, and the backbone of Met312. The α-COOH and γ-COOH groups make dipole-dipole interactions with water molecules. Compounds 13c-16c showed similar or better docking scores (-12.38 to -12.83 kcal/mol) compared to compound 46c (-12.38 kcal/mol), suggesting these compounds would display more potent inhibition of AICARFTase (Fig. 58).

The docking studies of 15c in the targets (Figures 55-58) displayed different conformational preferences of the side chain linker about the pyrrolo[2,3-d]pyrimidine scaffold. Superimposition of the docked poses of 15c was carried out in the crystal structures of FRs, GARFTase, and AICARFTase and enabled visualization of these conformations (Fig. 59). In reference to the scaffold, the side chains extend at different angles to bind to a particularly 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 for possible transport (FRs) and inhibition (GARFTase and/or AICARFTase).

**Figure 59.** Superposition of docked poses of 15c in crystal structures for FRα (grey), FRβ (pink), GARFTase (green) and AICARFTase (cyan).

**Figure 60.** Structure of proposed compounds in Series V

The Series IV (Fig. 53) was designed to investigate the influence of an intramolecular H-bond between the pyridyl nitrogen and the amide NH, whereas Series V (17c-19c, Fig. 60)
with the other regioisomer of the pyridyl ring was designed to evaluate influence of the pyridyl ring in the absence of the intramolecular H-bond on the antitumor activity and FRs/PCFT selectivity over RFC. Along with a lack of the intramolecular H-bond, varying the pyridyl regioisomer in 17c-19c versus the compounds 13c-16c alters the direction of the dipole moment of the pyridyl ring in the molecules which could result in different conformations and thereby different interaction with the binding sites. Compounds 17c-19c showed good docked scores in the binding sites of FRα (-14.68 to -15.17 kcal/mol), FRβ (-15.06 to -15.90 kcal/mol), GARFTase (-14.25 to -14.96 kcal/mol) and AICARFTase (-9.91 to -12.58 kcal/mol), suggesting these compounds would have potent antitumor activity.

B.2 Dual inhibitors of cytosolic (de novo purine biosynthetic) and mitochondrial (serine hydroxymethyltransferase 2) 1C metabolism enzymes

B.2.1. Design of 5-substituted, 2-amino-4-oxo pyrrolo[3,2-d]pyrimidine-pyridyl classical antifolates

Metabolic reprogramming is one of the hallmarks of cancer. 1C metabolism is dependent on an adequate supply of tetrahydrofolate (THF) metabolites to generate critical purine, thymidylate, and glycine metabolites for cell proliferation and tumor progression and it is a major pathway associated with the malignant phenotype. It is compartmentalized into the cytosol (de novo synthesis of thymidylate and purine nucleotides) and mitochondria (serine catabolism). Cytosolic and mitochondrial 1C metabolic pathways are interconnected by the exchange of serine, glycine, and formate. In cancer cells, serine is the major source of 1C units, and in mitochondria, serine catabolic enzymes including SHMT2, 5,10-methylene-THF dehydrogenase 2 (MTHFD2) and 10-formyl-THF synthetase (reverse) (MTHFD1L) generate glycine and 1C units (i.e., formate) to sustain 1C-dependent nucleotide and amino acid biosynthesis in the cytosol. 10-Formyl-THF is resynthesized from formate in the cytosol.
by the trifunctional enzyme MTHFD1. 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.

A study\textsuperscript{188} of messenger RNA profiles for over one thousand enzymes within nearly two thousand tumors across nineteen different cancer types identified SHMT2 and MTHFD2 among the top most upregulated genes. Thus, highlighting the key role of mitochondrial 1C metabolism across a wide spectrum of cancers. Metabolomic analyses of 219 extracellular metabolites from the NCI-60 cancer cell lines showed that glycine metabolism strongly correlated with cancer cell proliferation.\textsuperscript{309} Thus, mitochondrial 1C metabolism has been implicated as critical to the malignant phenotype.\textsuperscript{188, 309-311} These findings, combined with evidence of functional shortages of amino acids (\textit{e.g.}, glycine) in tumors,\textsuperscript{312} suggested a therapeutic opportunity for SHMT2 targeting in cancer.

Khushbu and Gangjee\textsuperscript{313} have reported the synthesis of a series of novel 5-substituted pyrrolo[3,2-\textit{d}]pyrimidine compounds (48c-53c, \textbf{Fig. 61}), which showed primarily transport via FR\textalpha{} and FR\textbeta{} with limited or no transport \textit{via} RFC and PCFT (\textbf{Table 6}).

\begin{center}
\begin{tabular}{ccc}
\textbf{Ar} & \textbf{n} & \textbf{Ar} \\
48c & 2 & 1,4-phenyl \\
49c & 3 & 1,4-phenyl \\
50c & 4 & 1,4-phenyl \\
51c & 2 & 2,5-thienyl \\
52c & 3 & 2,5-thienyl \\
53c & 4 & 2,5-thienyl \\
\end{tabular}
\end{center}

\textbf{Figure 61.} Structures of 5-Substituted Pyrrolo[3,2-\textit{d}]pyrimidine Classical Antifolates (48c-53c)
Table 9. IC<sub>50</sub> Values (nM) for 5-Substituted Pyrrolo[3,2-<i>d</i>]pyrimidine Classical Antifolates in RFC-, PCFT-, and FR-Expressing Cell Lines.

<table>
<thead>
<tr>
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<th>PC43-10</th>
<th>R2</th>
<th>RT16</th>
<th>D4</th>
<th>R2/hPCFT4</th>
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<td>1.59</td>
<td>25.2</td>
</tr>
<tr>
<td>&lt;i&gt;49c&lt;/i&gt;</td>
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<td>&gt;1000</td>
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<td>1.29</td>
<td>309</td>
</tr>
<tr>
<td>&lt;i&gt;50c&lt;/i&gt;</td>
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<td>&gt;1000</td>
<td>26.6</td>
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<td>&gt;1000</td>
<td>&gt;1000</td>
<td>14.62</td>
<td>7.89</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Table 10. Growth inhibition assays: IC<sub>50</sub> Values (nM) for 5-Substituted Pyrrolo[3,2-<i>d</i>]pyrimidines in KB human tumor sublines (expressing RFC, FRα, and PCFT) and protection study results by added metabolites.

<table>
<thead>
<tr>
<th></th>
<th>KB (nM)</th>
<th>Target</th>
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<tr>
<td>&lt;i&gt;48c&lt;/i&gt;</td>
<td>6.7</td>
<td>AICARFTase/SHMT2</td>
</tr>
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<td>&lt;i&gt;49c&lt;/i&gt;</td>
<td>6.8</td>
<td>GARFTase/AICARFTase/SHMT2</td>
</tr>
<tr>
<td>&lt;i&gt;50c&lt;/i&gt;</td>
<td>6.3</td>
<td>GARFTase/AICARFTase</td>
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<td>&lt;i&gt;53c&lt;/i&gt;</td>
<td>7.47</td>
<td>GARFTase/AICARFTase/SHMT2</td>
</tr>
</tbody>
</table>

Compounds <i>48c-53c</i> showed potent inhibition of KB tumor cell lines with inhibition of 1C metabolism cytosolic <i>de novo</i> purine biosynthesis (GARFTase and/or AICARFTase) and/or mitochondrial SHMT2 (Table 10). Inhibition of SHMT2 by classical antifolates has not been previously reported, and importantly this was achieved <i>via</i> scaffold hopping, as the
classical antifolates with the pyrrolo[2,3-d]pyrimidine scaffold, reported so far, have not been potent mitochondrial 1C enzyme inhibitors.

To determine the structural requirements for binding to the SHMT2 folate binding site, we docked 5-formyl THF (5-CHO-THF, resembles a natural substrate THF), 45c (pyrrolo[2,3-d]pyrimidine analog) and 48c (pyrrolo[3,2-d]pyrimidine analog) in the recently published\textsuperscript{314} SHMT2 crystal structure.

![Docked pose of 5-CHO-THF (white) in the SHMT2 folate binding site (PDB ID: 5V7I).](image)

Figure 62. Docked pose of 5-CHO-THF (white) in the SHMT2 folate binding site (PDB ID: 5V7I).

As there is no reported crystal structure of SHMT2 with classical folates, we docked 5-CHO-THF, which resembles the natural cofactor THF, to determine the binding requirements of SHMT2 binding site. Fig. 62 shows the docked pose of 5-CHO-THF in the SHMT2 binding site. The active site is located at the interface of chain A (green) and chain B (blue) and consists of a cavity at the bottom with a narrow channel opening into the solvent exposed area. The pteridine ring of 5-CHO-THF (docked score: -10.04 kcal/mol) binds in the
cavity with the para-amino benzoyl (PABA) ring binding in the narrow channel lined by Tyr176 and Tyr105, and the α-COOH makes a salt bridge with Lys181 whereas the γ-COOH makes ion-dipole interaction with the Ser178. The docked pose reveals an interesting conformation with the pteridine lying flat in the pocket with the PABA ring orthogonal to the pteridine scaffold. Based on the docked pose of 5-CHO-THF, it is hypothesized that for compounds to bind to SHMT2, they need to mimic this orthogonal conformation in the SHMT2 folate binding site.

Figure 63. Superposition of docked poses of 5-CHO-THF (white) and 45c (orange) in the SHMT2 folate binding site (PDB ID: 5V7I).

The docked pose of 5-substituted pyrrolo[2,3-\textit{d}]pyrimidine analog 45c in the SHMT2 cavity does not go as deep as the 5-CHO-THF (Fig. 63). Thus, the phenyl side-chain loses the π-π stacking interactions with Tyr105 and Tyr176. Therefore, the compound 45c shows a poor
docking score in the SHMT2 binding site (-7.42 kcal/mol) compared to 5-CHO-THF (-10.04 kcal/mol).

**Figure 64.** Superposition of docked poses of 5-CHO-THF (white) and 48c (cyan) in the SHMT2 folate binding site (PDB ID: 5V7I).

**Fig. 64** shows the superposition of 48c and 5-CHO-THF in the SHMT2 binding site, the pyrrolo[3,2-d]pyrimidine scaffold moves deeper in the pocket similar to 5-CHO-THF. This allows the phenyl side-chain to make the π-π stacking interactions with Tyr176 and the α- and γ-COOH groups to make salt bridges with Lys181 and Lys103, respectively. Compound 48c (-7.90 kcal/mol) showed a somewhat better docking score than the compound 45c (-7.42 kcal/mol) and similar interactions to the 5-CHO-THF.
Figure 65. Superposition of docked poses of 5-CHO-THF (white), 45c (orange) and 48c (cyan) in the SHMT2 folate binding site (PDB ID: 5V7I).

Superimposing the docked poses of 5-CHO-THF (white), 45c (orange) and 48c (cyan) clearly show the difference among the docked poses of 5-CHO-THF, 48c, and 45c (Fig. 65). The compound 48c with the side-chain phenyl ring attached to the pyrrole nitrogen of the pyrrolo[3,2-d]pyrimidine scaffold can mimic the orthogonal conformation of 5-CHO-THF, whereas 45c with the side-chain phenyl ring at the 5-position of the pyrrolo[2,3-d]pyrimidine scaffold is unable to adopt the same conformation. This unique feature of the pyrrolo[3,2-d]pyrimidine scaffold could be the reason that 48c can bind into the cavity of the SHMT2 binding site. Therefore, the pyrrolo[3,2-d]pyrimidine scaffold offers a unique opportunity to develop dual 1C 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 inhibition that would eliminate or decrease dose-limiting toxicity and would circumvent or delay the development of tumor resistance.
Figure 66. Series VI

Metabolite protection studies of 48c-53c (Table 10) implicate that not only the pyrrolo[3,2-d]pyrimidine scaffold but also the length of the linker and aromatic side-chain dictate the binding to various cellular targets. On the basis of this, compounds 20c-22c are proposed in Series VI (Fig. 66) with 3- (20c), 4- (21c), and 5-carbon (22c) linker analogs of 48c-50c with the pyridyl side-chain to evaluate the influence of the pyridyl ring, which can make intra- and intermolecular H-bonds in contrast to the phenyl ring along with other additional changes in molecule’s properties (Fig. 54), FRs/PCFT transport selectivity over RFC, multi-target engagement (GARFTase/AICAFTase/SHMT2) and on tumor cell inhibition.

Figure 67. Docked pose of 21c (magenta) in SHMT2 folate binding site (PDB: 5V7I).
The docked pose of compound 21c in the SHMT2 binding site is shown in Fig. 67. The pyrrolo[3,2-d]pyrimidine scaffold sits deep into the cavity similar to the 5-CHO-THF, the pyridyl ring makes π-π stacking interactions with Tyr105. The electron deficient pyridyl ring of 21c can make stronger π-π stacking interaction with Tyr105 than the phenyl side-chain in 48c. The α- and γ-COOH groups make salt bridges with Lys181 and Lys103, respectively. Compound 21c (-10.27 kcal/mol) showed higher docked score than the lead compound 48c (-7.90 kcal/mol), suggesting that it would be a more potent inhibitor of SHMT2 than the lead 48c. Three carbon (20c, -8.82 kcal/mol) and five carbon (22c, -9.56 kcal/mol) linker analogs also showed good docked scores in the SHMT2 binding site. Compounds 20c-22c also showed good docked scores in the transporters; FRα (-15.67 to -15.79 kcal/mol) and FRβ (-14.06 to -15.13 kcal/mol) and de novo purine biosynthesis enzymes; GARFTase (-13.98 to -15.01 kcal/mol), and AICARFTase (-11.27 to -12.39 kcal/mol). Thus, compounds 20c-22c are proposed as potent inhibitors of both cytosolic and mitochondrial 1C metabolism enzymes.

B.2.2. Design of 5-substituted, 2-amino-4-oxo pyrrolo[3,2-d]pyrimidine-3’-fluoropyridyl classical antifolates

Bioisosteric replacement of the hydrogen atom with the fluorine atom in bioactive molecules has been successfully used for modifying the biological properties of drugs. FDA-approved fluorinated drugs increased in past decade (from 20% in 2010 to about 30% currently).\textsuperscript{315, 316} Fluorine is ~20% larger than hydrogen (van der Waals radii), the C–F bond is longer and larger compared to a C–H bond, has a larger dipole moment in the opposite direction of a C–H, is modestly more lipophilic and can form strong electrostatic interactions including fluorine-hydrogen bonds (Table 11).\textsuperscript{317-325} Fluorine is also a high impact design element, owing to its multitude of effects, can be used for multi-parameter optimization.
Table 11. Properties and application of the C-F bond compared to the C-H bond.\textsuperscript{324}

<table>
<thead>
<tr>
<th>Properties</th>
<th>C-H</th>
<th>C-F</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond Length (Å)</td>
<td>1.09</td>
<td>1.35</td>
<td>Hydrophobic interactions, lipophilicity, conformational effects</td>
</tr>
<tr>
<td>Total size (Å(^3))</td>
<td>2.29</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td>Electronegativity of H/F</td>
<td>2.2</td>
<td>3.98</td>
<td>Non-covalent interactions, steric/electronic effects, conformational effects</td>
</tr>
<tr>
<td>Dipole moment (µ)</td>
<td>-0.4</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 68](image)

**Figure 68.** Previously synthesized pyrrolo[2,3-\(d\)/[3,2-\(d\)]pyrimidine classical antifolates.

Ravindra \textit{et al.}\textsuperscript{326} recently reported the influence of 2’-fluorine (\textit{ortho} to \textit{l}-glutamate group) substitution in the phenyl side-chain ring on the transporter selectivity (FRs/PCFT over RFC) and tumor cell inhibition of 2-amino-4-oxo-6-substituted pyrrolo[2,3-\(d\)]pyrimidine antifolates (\textit{54c-57c}, \textit{Fig. 68}). Junayed and Gangjee\textsuperscript{327} also evaluated the influence of replacement of the hydrogen with a fluorine atom on the phenyl side-chain of 2-amino-4-oxo-5-substituted pyrrolo[3,2-\(d\)]pyrimidine antifolates (\textit{58c-61c}, \textit{Fig. 68}) with dual cytosolic and mitochondrial 1C metabolism enzyme inhibition.
Table 12. IC₅₀ Values (nM) for classical antifolates with the phenyl and fluorinated-phenyl side-chain in RFC-, PCFT-, and FR-expressing cell lines and KB tumor cell line.

<table>
<thead>
<tr>
<th></th>
<th>hRFC PC43-10</th>
<th>R2</th>
<th>hFRα RT16</th>
<th>hFRβ D4</th>
<th>hPCFT R2/hPCFT4</th>
<th>KB (IC₅₀s) nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>54c</td>
<td>304</td>
<td>448</td>
<td>4.1</td>
<td>5.6</td>
<td>23</td>
<td>1.8</td>
</tr>
<tr>
<td>56c</td>
<td>62</td>
<td>140</td>
<td>1.12</td>
<td>3.87</td>
<td>3.82</td>
<td>2.6</td>
</tr>
<tr>
<td>55c</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>6.3</td>
<td>6.3</td>
<td>213</td>
<td>1.6</td>
</tr>
<tr>
<td>57c</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.58</td>
<td>1.6</td>
<td>23</td>
<td>0.59</td>
</tr>
<tr>
<td>58c</td>
<td>43</td>
<td>&gt;1000</td>
<td>50</td>
<td>1.59</td>
<td>25.2</td>
<td>6.7</td>
</tr>
<tr>
<td>60c</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>22.89</td>
<td>0.52</td>
<td>155</td>
<td>9.07</td>
</tr>
<tr>
<td>59c</td>
<td>516</td>
<td>&gt;1000</td>
<td>2.13</td>
<td>1.29</td>
<td>309</td>
<td>6.8</td>
</tr>
<tr>
<td>61c</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.8</td>
<td>1.03</td>
<td>&gt;1000</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Note: The fluoro analogs highlighted with blue color fonts.

Interestingly, the fluorine atom had a variable influence on the biological activities of antifolates depending on the scaffold and linker length (Table 12). Fluorine increased inhibition of CHO cells with transport by FRα and FRβ compared to the des-fluoro analogs. The pyrrolo[2,3-d]pyrimidine analogs (56c and 57c) showed an increase in inhibition of PCFT-expressing CHO cell lines compared to 54c and 55c, whereas the pyrrolo[3,2-d]pyrimidine analogs (60c and 61c) showed the reverse trend upon fluorine substitution. The fluoro analogs showed similar or better potency towards the KB tumor cell lines and completely lost RFC transport, except for compound 56c, compared to their corresponding des-fluoro analogs. These results indicated that the fluorine was conducive to selective transport via FR and PCFT over RFC.

We designed compounds 23c and 24c (Fig. 69) to evaluate the effects of the fluorine atom on transporter selectivity and tumor cell inhibition of the compounds 21c and 22c. The pyridyl side-chain in 21c and 22c can intramolecularly H-bond with the amide NH, resulting in conformational restriction, whereas in compounds 23c and 24c the conformational
restriction can occur either due to the pyridyl nitrogen or the fluorine atom making intramolecular H-bonds with the amide NH or an unfavorable electronic repulsion with the lone pairs of the carbonyl oxygen. Also, the fluorine atom can make H-bond or other dipole-dipole interactions in the binding pockets of the various targets.

Figure 69. Series VII.

Figure 70. A) Superimposition of the conformations of 23c produced by a coordinate scan and B) Plot of the relative energy of the conformations (kcal/mol) versus the N1-C2-C3-O4 dihedral angle (degree).
A coordinate scan around the N1-C2-C3-O4 dihedral angle (Fig. 70A) was performed to determine the rotational barrier and water as a solvent to perform the calculations to mimic physiological conditions using Maestro. Fig. 70A shows the superimposition of all the conformations of 23c, with the minimum energy conformation shown in cyan (dihedral angle: 50 °) and the second lowest energy conformation in pink (dihedral angle: -50.1 °) both adopt gauche conformation around the N1-C2-C3-O4 which suggests that electronic repulsion effect dominates over intra-molecular H-bond interaction, which requires a dihedral angle of 0 ° or 180 °. The plot indicates the energy barrier of ~4 kcal/mol between the two minima, represented by a dihedral angle of 180 °, the conformation in which fluorine and carbonyl oxygen are in the same plane and on the same side affords the most unfavorable electronic repulsion. The energy barrier predicted by the calculations in Fig. 70A further corroborates our hypothesis that introduction of fluorine can have several effects, one of which is a conformational restriction. Thus, compounds with a 3’-fluoropyridyl side-chain (23c and 24c) are predicted to adopt different conformations than the compounds with just the pyridyl side-chain (20c-22c) or phenyl side-chains (60c and 61c).

The docked poses of compounds 23c and 24c in the SHMT2 binding site are shown in Figure 71. The pyrrolo[3,2-d]pyrimidine scaffold sits deep into the cavity similar to 5-CHO-THF, the 3’-fluoropyridyl ring occupies the narrow channel lined by Tyr105 and Tyr176, the amide group makes H-bonds with Tyr105 and Tyr176. The relative conformations of the pyridyl ring and the amide group in the docked conformations of 23c and 24c in the SHMT2 binding site are gauche (N1-C2-C3-O4 dihedral angle: -71° and 90°) which are closer to the minimum energy conformations of these molecules. Thus, it can provide entropic benefit to these compounds when bound in the SHMT2 folate binding site. The compounds 23c and 24c also showed good docked scores in the folate transporters; FRα (-15.55 and -15.12 kcal/mol, respectively) and FRβ (-15.89 and -15.80 kcal/mol, respectively) and de novo purine
biosynthesis enzymes; GARFTase (-14.08 and -13.79 kcal/mol, respectively), and AICARFTase (-10.89 to -11.99 kcal/mol). Thus, 23c and 24c are proposed to be potent inhibitors of both cytosolic and mitochondrial 1C metabolism enzymes.

**Figure 71.** Docked poses of 23c (cyan) and 24c (orange) in SHMT2 folate binding site (PDB: 5V7I).

**B.3 Design of 5-substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-d]/[3,2-d]pyrimidine-pyridyl classical antifolates**

Although the methyl group, often considered as chemically inert, only participates in London dispersion interactions, it plays a very important role in the molecular recognition of endogenous and exogenous ligands by bioreceptors. The stereoelectronic effects of a methyl leads to selective binding and increased potency, among various other pharmacological effects. As such, it is a useful tool employed in medicinal chemistry design to modify
aspects related to conformation, electronic factors, pharmacokinetics, among other factors.\textsuperscript{330, 331}

Studies of 5- and 6-substituted pyrrolo[2,3-\textit{d}]pyrimidines established that while the 6-substituted analogs are selective (>100 fold for FR and/or PCFT expressing CHO cells over those that express RFC) and potent GARFTase inhibitors, the related 5-substituted pyrrolo[2,3-\textit{d}]pyrimidines are non-selective (<10 fold for FR and/or PCFT expressing CHO cells over those that express RFC) dual GARFTase and AICARFTase inhibitors.\textsuperscript{175, 181, 185, 332, 333} A superior inhibitor to the above two series of analogs would be a compound that is FR- and PCFT-selective (over RFC) and dual GARFTase and AICARFTase inhibitor. Thus, it was of interest to synthesize and evaluate 6-methylated, 2-amino-4-oxo-pyrrolo[2,3-\textit{d}]pyrimidines 64\textit{c}, 65\textit{c}, and 66\textit{c} as the hybrids of 5- and 6-substituted analogs, to evaluate for improved selectivity and dual enzyme inhibitory activity (Fig. 72).

\begin{center}
\textbf{Figure 72.} Lead compounds 62\textit{c}, 63\textit{c}, PMX, 45\textit{c}, 46\textit{c}, 64\textit{c}, 65\textit{c} and 66\textit{c}.
\end{center}
Table 13. IC\textsubscript{50} Values (nM) and FR-and PCFT-expressing CHO cell selectivity ratios over RFC-expressing CHO cells. For KB cells, data are shown for the protective effects of nucleoside additions, including adenosine (Ade), thymidine (Thd), or AICA.

<table>
<thead>
<tr>
<th></th>
<th>RFC/FR\textalpha Selectivity ratios (IC\textsubscript{50}) for FR\textbeta over RFC</th>
<th>RFC/FR\textbeta Selectivity Ratios (IC\textsubscript{50}) for FR\textbeta over RFC</th>
<th>RFC/PCFT Selectivity ratios (IC\textsubscript{50}) for PCFT over RFC</th>
<th>KB (+Thd/Ade/AICA)</th>
<th>KB (IC\textsubscript{50}) nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMX</td>
<td>0.62</td>
<td>0.44</td>
<td>3.15</td>
<td>Thd/Ade</td>
<td>9.94±3.11</td>
</tr>
<tr>
<td>45c</td>
<td>0.95</td>
<td>-</td>
<td>0.2</td>
<td>Ade</td>
<td>49.5±13.2</td>
</tr>
<tr>
<td>65c</td>
<td>&gt;15</td>
<td>&gt;385</td>
<td>&gt;17</td>
<td>Ade/AICA/Thd</td>
<td>49.9</td>
</tr>
</tbody>
</table>

Gangjee et al\textsuperscript{334} performed a study of a 5-substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-d]pyrimidine 57 with a 3C-linker length and found that the hypothesis for selectivity due to a 6-methyl substitution was supported by the biological activity. Compound 65c is a multi-enzyme inhibitor of purine and pyrimidine biosynthesis (Table 13); in vitro growth inhibition of KB tumor cells was completely protected only in the presence of both Ade (adenine) and Thd (thymidine) (Table 13). The potency of compound 65c towards KB cells (49.9 nM) was identical to its parent desmethyl analog 45c (Table 13). Unlike 45c (equally potent for RFC- and FR\textalpha-expressing CHO cells, and ~5-fold selective for RFC- over PCFT-expressing CHO cells), 65c is selective for FR\textalpha, FR\textbeta and PCFT over RFC- expressing CHO cells (>15-, >385- and >17-fold, respectively) primarily due to loss of uptake by RFC (Table 13). This study led to the discovery of a compound that was ~5-fold less potent against KB cell proliferation and ~5-(PCFT) to 900-(FR\textbeta) fold more selective than PMX. The pharmacological influences of a methyl substitution for the observed loss of RFC binding is perhaps due to steric repulsion. The crystal structure of hRFC is not known, and hence, the arrangement of the amino acids that line the folate binding pocket and the binding interactions remain unidentified. However, based on the general trend of reduced RFC binding by the 6-substituted pyrrolo[2,3-
pyrimidine series of analogs, a possible steric repulsion in the scaffold binding subsite caused by the alkyl group at 6-position is proposed.

To investigate the contribution of 6-methyl substitution in the pyridyl series of 5-substituted pyrrolo[2,3-\textit{d}] and [3,2-\textit{d}]pyrimidine analogs for improved potency as well as selectivity, compounds 25c and 26c with 3C- (6-methyl analog of 14c) and 4C-linkers (6-methyl analog of 21c), respectively were designed (\textbf{Fig. 73}).

\textbf{Figure 73.} Target compounds 25c and 26c, Series VIII.

Molecular modeling studies of the 6-methylated analogs 25c-26c were carried out using X-ray crystal structures of human FR\(\alpha\) (5IZQ), and GARFTase (4ZZ1) to explore the binding interactions and to validate their drug targets. The compounds display similar interactions as the native crystal structure ligands (not shown here for clarity).

The docked poses of the 25c (-15.23 kcal/mol) and 26c (-16.18 kcal/mol) in FR\(\alpha\) (\textbf{Fig. 74}) display similar interactions as the native crystal structure ligands (not shown here for clarity) by maintaining key interactions involving the bicyclic scaffolds and the pyridyl L-glutamate tail. The 6-methyl substitution neither sterically clashes with the binding site of FR\(\alpha\), nor does it alter the conformation of the scaffold when compared to the desmethyl analogs. Additionally, the 6-methyl group orients towards hydrophobic amino acid residues such as Leu 59, and Phe 62, which can increase binding to the FR\(\alpha\) binding site.
Figure 74. A) Docked pose of 25c (orange) and B) 26c (cyan) in the FRα binding site (PDB ID: 5IZQ).

Unlike the extended pocket of the FRs, the binding pocket in GARFTase is curved and forces a conformation such that the linker projects the sidechain aryl ring almost at a 90° angle. As such, the binding pocket accommodates and maintains key interactions with classical folate analogs that have linkers ranging from 2-atoms to 5-atoms. The 6-methyl analog 25c can accommodate the bent conformation similar to other classical antifolates with the 6-Me oriented toward hydrophobic residue Leu 892 whereas, the 6-Me in 26c forces the scaffold to bind in a flipped orientation (Fig. 75). Although 25c (-14.53 kcal/mol) shows a better docking score than 26c (-13.70 kcal/mol), the docking scores are within the range to predict good GARFTase inhibition.
Figure 75. A) Docked pose of 25c (orange) and B) 26c (cyan) in the GARFTase binding site (PDB ID: 4ZZ1).

C. Selective inhibition of pjDHFR

One of the most efficient strategies to treat PCP infection is to target *Pneumocystis jirovecii* DHFR (pjDHFR).\(^{220}\) DHFR catalyzes the reduction of 7,8-dihydrofolate to the 5,6,7,8-tetrahydrofolate. Inhibition of DHFR interferes with thymidylate biosynthesis and consequently DNA synthesis, and the inhibition of folate-dependent formyl transferases
causing inhibition of purine synthesis as well. This inhibition disrupts DNA, RNA, and protein synthesis of the organism and eventually leads to the death of the organism.

**Figure 76.** Superimposition of active sites of pcDHFR (pink color) and pjDHFR (green color), co-crystallized with the ligand $N^6$-methyl-$N^6$-(naphthalen-2-yl)pyrido[2,3-$d$]pyrimidine-2,4,6-triamine (cyan) represent the active site of pcDHFR (PDB: 4QJZ, 1.61 Å).

Pneumocystis infection is host-species specific. Most of the drugs synthesized and evaluated thus far for PCP infections were tested against *Pneumocystis carinii* DHFR (pcDHFR), which was presumed to be the causative species of PCP infection in humans. However, *P. carinii*, however, is a distinct species that infects rats, different from *P. jirovecii*, responsible for human infections. The amino acid sequence of the DHFR of *P. carinii* (pcDHFR) differs by 38% when compared to the DHFR of *P. jirovecii* (pjDHFR). Isolation of pjDHFR along with the development of a homology model for pjDHFR provided the amino-acid sequence differences between pjDHFR and pcDHFR, as well as hDHFR. The superimposition of the active site of pcDHFR and pjDHFR (Fig. 76) displays the amino acid differences present in the active sites of the two enzymes and underscores the futility of designing and evaluating activity against the surrogate pcDHFR as inhibitors of pjDHFR. Hence, drugs evaluated against
the surrogate pcDHFR \textit{in vitro} may not translate into activity in the treatment of PCP infection in humans.

**Table 14.** Inhibition Concentrations (IC\textsubscript{50}) against pjDHFR and hDHFR and Selectivity Ratios.

<table>
<thead>
<tr>
<th></th>
<th>pjDHFR (nM)</th>
<th>hDHFR (nM)</th>
<th>Selectivity Ratios [hDHFR/pjDHFR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMP (27c)</td>
<td>92</td>
<td>245000</td>
<td>266</td>
</tr>
<tr>
<td>PTX</td>
<td>41</td>
<td>2</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The recently isolated pjDHFR\textsuperscript{221} has been used to evaluate clinically used agents such as TMP (trimethoprim), PTX (piritrexim), and novel DHFR inhibitors\textsuperscript{222}. These studies demonstrated that the inhibition of human(h)DHFR compared with pjDHFR allows a selectivity ratio (IC\textsubscript{50} hDHFR/ IC\textsubscript{50} pjDHFR) that provides a measure of the selectivity of the agent for pjDHFR over hDHFR. Compounds, such as PTX and TMQ (trimetrexate) due to low selectivity for pjDHFR over hDHFR and are much too 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 (Table 14). However, besides the selectivity for pjDHFR, another aspect that is highly desirable is for selective agents potency for pjDHFR. TMP is a poor inhibitor of pjDHFR and lacks single agent \textit{in vivo} efficacy against parasitic infections and must be used with SMX. The long-term goal is 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 PCP infections in humans.

Rational design of pjDHFR inhibitors is hampered due to a lack of X-ray crystal structure of pjDHFR. However, homology models can be used with refinement to model pjDHFR in the absence of crystal structures.\textsuperscript{222} Thus, along with known hDHFR X-ray crystal structures,\textsuperscript{223} pjDHFR homology models can be used to design potent and selective pjDHFR inhibitors. Another significant impediment in the drug discovery of inhibitors of pjDHFR is
the inability to grow the organism outside the human lung, and hence it is difficult to develop a tissue culture for in vitro studies or an animal model for \textit{in vivo} evaluation of the synthesized compounds. Due to this drawback, isolation and use of the pjDHFR enzyme is currently the only direct indicator that a compound could be tested for the treatment of PCP infection in humans.

\textbf{Figure 77.} Superimposition of active sites of hDHFR (pink color) and pjDHFR (green color), co-crystallized with the ligand $N^6$-methyl-$N^6$-(3,4,5-trifluorophenyl)pyrido[2,3-$d$]pyrimidine-$2,4,6$-triamine (magenta) (cyan) represents the active site of hDHFR (PDB: 4QJC, 1.62 Å).

To study the differences in the active site of pjDHFR and hDHFR, the pjDHFR homology model sequence was superimposed on the hDHFR X-ray crystal published with pyrido[2,3-$d$]pyrimidines (\textbf{Fig. 77}).\textsuperscript{222, 223} The amino acid differences are displayed. The active site of hDHFR is composed of Phe31, Ile60, Asn64, and Val115. The corresponding residues in the active site of pjDHFR are composed of Met33, Leu65, Ser69, and Ile123. These amino acids possess varied shapes, sizes, and electrostatic properties, which display different bindings to a potential ligand and can be exploited for selectivity and potency over hDHFR.
**Figure 78.** Lead compound trimethoprim (TMP) and target compounds 28c-32c, Series IX.

**Figure 79.** Docked pose 29c (orange) in (A) homology model of pjDHFR (-10.27 kcal/mol) and (B) crystal structure of hDHFR (-8.56 kcal/mol) (PDB: 4QJC, 1.62 Å)

The pocket where the side chain aryl group binds is partially composed of Met33, Pro66, Leu65, Ser64, and Ile123 in pjDHFR and Phe31, Pro61, Ile60, Ser59, and Val115 in hDHFR. Thus, the two active sites have different properties of electrostatics, shape, and size.

To achieve potency and selectivity by targeting these differing residues, the side-chain aryl substituents with electron withdrawing, and electron donating groups in place of the 3',4',5'-trimethoxyphenyl group in TMP were chosen for Series IX (compounds 28c-32c, Fig. 79).

Gangjee and coworkers$^{194, 222}$ have previously showed that changes in the side-chain aryl substitution are an effective strategy to improve potency and selectivity for pjDHFR over hDHFR.
Figure 80. Docked pose of 32c (cyan) in (A) homology model of pjDHFR (-10.34 kcal/mol) and (B) crystal structure of hDHFR (-8.91 kcal/mol) (PDB: 4QJC, 1.62 Å)

All the proposed compounds displayed favorable docking scores for pjDHFR (-10.09 to -10.37 kcal/mol) over hDHFR (-8.39 to -8.91 kcal/mol), similar or better than the lead compound TMP (pjDHFR; -10.02 kcal/mol and hDHFR; -8.33 kcal/mol) (Fig. 79 and 80).
IV. CHEMICAL DISCUSSION

The present work deals with the development of synthesis in the following three major areas:

D. Synthesis of single agents with combination chemotherapy potential

A.1. Synthesis of multiple receptor tyrosine kinases inhibitors and microtubule targeting agents:
    Quinazoline and Pyrido[3,2-\textit{d}]pyrimidine analogs as multiple receptor tyrosine kinase inhibitors and microtubule targeting agents

A.2. Synthesis of single agents with TS and multiple tyrosine kinase inhibitors:
    Pyrimido[4,5-b]indoles as receptor tyrosine kinase and thymidylate synthase inhibitors

B. Synthesis of folate Receptors (FR) and/or proton-coupled folate transporter (PCFT) targeted (cytosolic and/or mitochondrial) one-carbon (1C) metabolism inhibitors

B.1. Synthesis of inhibitors of \textit{de novo} purine biosynthetic enzymes (cytosolic 1C metabolism)
    1. 5-Substituted, 2-amino-4-oxo pyrrolo[2,3-\textit{d}]pyrimidine-pyridyl classical antifolates

B.2. Synthesis of dual inhibitors of cytosolic (\textit{de novo} purine biosynthetic) and mitochondrial (serine hydroxymethyltransferase 2) 1C metabolism enzymes
    1. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-\textit{d}]pyrimidine-pyridyl classical antifolates
    2. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-\textit{d}]pyrimidine-3'-fluoropyridyl classical antifolates

B.3. Synthesis of 5-substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-\textit{d}]/[3,2-\textit{d}]pyrimidine-pyridyl classical antifolates

C. Synthesis of selective inhibition of \textit{P. jirovecii} dihydrofolate reductase

2. 5-substituted, 2,4-diamino pyrimidines (trimethoprim analogs) as selective pjDHFR inhibitors
A. Synthesis of single agents with combination chemotherapy potential

A.1. Synthesis of multiple tyrosine kinases inhibitors and microtubule targeting agents:
Quinazoline and Pyrido[3,2-d]pyrimidine analogs as multiple tyrosine kinase inhibitors and microtubule targeting agents

**Scheme 41:** Synthesis of target compound 1c (Series I)

4-(methylthio)aniline (1d) (**Scheme 41**) was reacted with paraformaldehyde and sodium methoxide in methanol at rt for 5 h, then sodium borohydride was added, and the solution was heated to reflux for 2 h to provide N-methyl-4-(methylthio)aniline (2d) in 89% yield.

Displacement of the 4-chloro of 2,4-dichloroquinazoline (3d) with 2d in acetonitrile provided final compound 2-chloro-N-methyl-N-(4-(methylthio)phenyl)quinazolin-4-amine (1c) in 22% yield.

Monomethylated aniline, 5-methoxy-N-methylnaphthalen-2-amine (7d, **Scheme 42**), was synthesized over three steps from 6-aminonaphthalen-1-ol (4d). Boc protection of 4d in THF at rt yielded tert-butyl (5-hydroxynaphthalen-2-yl)carbamate (5d) in 86% yield over 4 hours. Compound 5d was methylated using 2.1 eq of methyl iodide to give tert-butyl (5-methoxynaphthalen-2-yl)(methyl)carbamate (6d) in 90% yield. Finally, Boc deprotection using trifluoroacetic acid (TFA) provided 5-methoxy-N-methylnaphthalen-2-amine (7d) in 93% yield. Commercially available quinazolin-4(3H)-one (8d) (**Scheme 42**) was reacted with stoichiometric amounts of phosphorous oxychloride (POCl₃) and triethylamine in toluene to
produce 4-chloroquinazoline (9d) in 67% yield. To reduce side-products care was taken during work-up that ice-cold solutions of ethyl acetate, water, sat. NaHCO₃, 1N HCl and brine were used. S_N AR displacement of the 4-chloro of 9d with aniline 7d in acetonitrile using a drop of conc. HCl as catalyst provided the final compound N-(5-methoxynaphthalen-2-yl)-N-methylquinazolin-4-amine (2c) in 85% yield as a yellow solid.

**Scheme 42:** Synthesis of target compound 2c (Series I)

3-Aminopicolinic acid (10d) (**Scheme 43**) was cyclized with urea at 160 °C overnight to afford pyrido[3,2-d]pyrimidine-2,4(1H,3H)-dione (11d) in 60% yield. Dichlorination of 11d using stoichiometric amounts of POCl₃ (2.2 eq) gave 2,4-dichloropyrido[3,2-d]pyrimidine (12d) in 68% yield. Displacement of the 4-chloro of 12d with the corresponding anilines in acetonitrile using catalytic amount of conc. HCl gave final compounds 3c – 6c as yellow solids.
in 44-69% yields (Compound 3c = 55%, compound 4c = 44%, compound 5c = 48% and compound 6c = 69%).

**Scheme 43:** Synthesis of target compounds 3c - 6c.

The carbon ($^{13}$C) NMR spectra of the final compounds (3c-6c) were used to confirm the regioselectivity of the S$_{N}$Ar reaction at 4-position vs 2-position of 12d. To establish the structures of the final compounds, compound 6c (synthesis and X-ray crystal structure of it was recently published$^{340}$) was used as a template by comparing the $^{13}$C spectrum of 6c reported in literature with our experimental spectrum. Literature$^{340}$ $^{13}$C NMR (DMSO-d$_6$) $\delta$ 161.36, 156.39, 155.56, 148.63, 147.74, 134.99, 133.57, 132.68, 132.61, 128.45, 124.98, 112.48, 111.65, 55.15, 48.48, 25.97, 23.58. Experimental $^{13}$C NMR (500 MHz, DMSO-d$_6$) $\delta$ 161.86, 156.90, 156.07, 149.13, 148.24, 135.49, 134.07, 133.18, 133.12, 128.95, 125.48, 112.98, 112.14, 55.65, 48.99, 26.47, 24.08.
Scheme 44: Synthesis of target compounds 7c – 10c

3-Aminopicolinic acid (10d) (Scheme 44) was cyclized with formamide (13d) at 170 °C in the microwave reactor for 1 h to afford pyrido[3,2-d]pyrimidin-4(3H)-one (14d) in 56% yield. Chlorination of 14d using stoichiometric amount of POCl₃ gave 4-chloropyrido[3,2-d]pyrimidine (15d) in 70% yield. Displacement of 4-chloro of 15d with the corresponding anilines in acetonitrile using catalytic amounts of conc. HCl gave the final compounds 7c – 10c as yellow solids in 39-93% yields (7c = 52%, 8c = 48%, 9c = 39% and 10c = 93%).
A.2. Synthesis of single agents with TS and multiple tyrosine kinase inhibitors:

Pyrimido[4,5-b]indoles as receptor tyrosine kinase and thymidylate synthase inhibitors

**Scheme 45**: Retrosynthetic scheme of lead compounds.

The lead compounds were synthesized previously^{123} using the retrosynthetic scheme presented above (**Scheme 45**). Nucleophilic aromatic substitution of ethyl cyanoacetate with commercially available 1,2-dichloro-3-nitrobenzene (H), and reductive cyclization to afford ethyl 2-amino-4-chloro-1H-indole-3-carboxylate (F). Cyclization of F with chlorformamidine to give tricyclic 2-amino-5-chloro-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (E). The 2-amino group was protected with the pivaloyl group to afford N-(5-chloro-4-oxo-4,9-dihydro-3H-pyrimido[4,5-b]indol-2-yl)pivalamide (D). Chlorination and amination provided 5-chloro-
9H-pyrimido[4,5-b]indole-2,4-diamine (B) and final S-N AR displacement of the 5-chloro of B at 250 °C with respective aryl thiols provided the lead compounds (A1-A2).

Scheme 46: Synthesis of target compounds (11c and 12c)

The synthesis of target compounds (11c and 12c) (Scheme 46) was envisioned via a nucleophilic aromatic substitution reaction in the first step of the lead compounds (Scheme 45) but with different starting materials, malononitrile and 1-bromo-2-fluoro-3-nitrobenzene (16d), instead of 1,2-dichloro-3-nitrobenzene (H) and ethyl cyanoacetate. This change was proposed to increase the reactivity of the 2-position at the aromatic ring with fluorine instead of chlorine. Switching to malononitrile from ethyl cyanoacetate removed the need for multiple steps to convert the 4-oxo to 4-amino group, reducing the number of steps. The reaction of 16d with malononitrile gave 2-(2-bromo-6-nitrophenyl)malononitrile (17d) in 78% yield. Reductive cyclization of 17d with zinc in acetic acid at 55 °C afforded 2-amino-4-bromo-1H-indole-3-carbonitrile (18d) in 60% yield and cyclization with chlorformamidine HCl in methyl sulfone at 120 °C overnight yielded 5-bromo-9H-pyrimido[4,5-b]indole-2,4-diamine (19d) in 45% yield. Another crucial change in the synthesis of the target compounds was the C-O bond
formation at the 5-position of 19d. Direct nucleophilic displacement was initially attempted but it resulted in mainly the dehalogenated product and degradation side-products. This was due to the poor nucleophilic nature of the phenoxide compared to the aryl thiols. Ullmann-type copper-catalyzed couplings have been successfully used for C-O bond formation.\textsuperscript{342} Ullmann coupling of 19d with corresponding sodium phenoxides with copper iodide and potassium carbonate were tried at different temperatures (130-180 °C) but only trace amounts of the products were observed with the dehalogenated product as the major product on TLC.

Table 15: Optimization of Ullmann Coupling conditions.

<table>
<thead>
<tr>
<th>#</th>
<th>Ligand</th>
<th>Base</th>
<th>Reaction Progress (% conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="L1" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>Yes (50%)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="L2" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="L3" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="L4" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="L5" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>Yes (20%)</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="L6" /></td>
<td>K\textsubscript{2}CO\textsubscript{3}</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="L7" /></td>
<td>Cs\textsubscript{2}CO\textsubscript{3}</td>
<td>Yes (50%)</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8.png" alt="L8" /></td>
<td>Cs\textsubscript{2}CO\textsubscript{3}</td>
<td>Yes (10%)</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9.png" alt="L9" /></td>
<td>Cs\textsubscript{2}CO\textsubscript{3}</td>
<td>Yes (90%)</td>
</tr>
</tbody>
</table>
Addition of ligands have been reported to improve yield and decrease dehalogenated products in these types of coupling reactions. Several roles have been assigned to ligands such as increasing solubility of copper reagents, and facilitating various stages of the coupling i.e. oxidative-addition or reductive-elimination. The most successful ligands are of two types: neutral (neocuproine \( \text{L1} \), \( \text{N,N-dimethylene-1,2-diamine L4} \), and 2-aminopyridine \( \text{L5} \)) or anionic (dimethylglycine \( \text{L2} \), quinolin-8-ol \( \text{L3} \) and 2-isobutyrylcyclohexan-1-one \( \text{L6} \) (Table 15). Thus these ligands were implemented in the Ullmann coupling to synthesize the final compound \( 11c \). First, these ligands were used with potassium carbonate (base) under previously reported conditions (180 °C, Scheme 46). The reaction progress was observed with only two ligands \( \text{L1} \) (50% conversion) and \( \text{L5} \) (20% conversion). The rest of the ligands showed no trace of product after 1 h of reaction. Neutral ligands have been especially successful in formation of C-O bond with Ullmann coupling. Copper catalysts ligated with neutral ligands need to coordinate with nucleophiles to facilitate oxidative addition to the aryl-halide bond (rate-determining step) and coordination of copper metal with the oxygen nucleophile is highly favored. As the nucleophile is already coordinated to the catalyst while it undergoes oxidative-addition into the aryl-halide bond this further accelerates reductive-elimination. The presence of neutral ligands also reduces the formation of dehalogenated side-product. For further optimization potassium carbonate was substituted with cesium carbonate as cesium ions can improve solubility of various copper species (Cu(I) and Cu(III)) involved in the catalytic cycle. Indeed, cesium carbonate showed significant improvement in the reaction progress with conversions reaching almost 90% with ligand \( \text{L5} \) (Table 15).
**B. Synthesis of folate Receptors (FR) and/or proton-coupled folate transporter (PCFT) targeted (cytosolic and/or mitochondrial) one-carbon (1C) metabolism inhibitors**

**B.1. Synthesis of inhibitors of de novo purine biosynthetic enzymes (cytosolic 1C metabolism)**

1. 5-Substituted, 2-amino-4-oxo pyrrolo[2,3-d]pyrimidine-pyridyl classical antifolates

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**Scheme 47:** Optimized Ullmann coupling for synthesis of 11c and 12c

The optimized coupling condition with ligand 24d (Scheme 47) and cesium carbonate as base was used to synthesize the final compounds 11c and 12c in 70% and 68% yield, respectively.

**Scheme 48:** Synthesis of intermediate aldehyde 22d.

---
Esterification of 5-bromopicolinic acid (20d) (Scheme 48) using the reported procedure produced ethyl 5-bromopicolinate (21d) as a white solid in 80% yield. To synthesize the key intermediate ethyl 5-(5-oxopentyl)picolinate (22d) for the synthesis of the final compound 14c, initially a Heck coupling (condition A, Scheme 48) between 21d and pent-4-en-1-ol was attempted which produced too many impurities and afforded 22d in only 10% yield as a clear oil. Alternatively, Sonogashira coupling of 21d and pent-4-yn-1-ol using palladium chloride, triphenyl phosphine, triethyl amine and copper iodide at 100 °C in a microwave reactor yielded ethyl 5-(5-hydroxypent-1-yn-1-yl)picolinate (23d) in 70% yield. Palladium-catalyzed reduction of the alkyne and oxidation of the alcohol using Dess-Martin periodinane yielded intermediate aldehyde 22d as a clear oil, in better yield (~42%) over three steps from 21d to 22d compared to the Heck coupling (~10%). Therefore, to synthesize the intermediate aldehydes this three step route (condition B) was utilized.

Scheme 49: Synthesis of compound 14c.
First, α-bromination of the aldehyde 22d (Scheme 49) with bromine (Br$_2$) in 1,4-dioxane at room temperature was attempted. This afforded a more non-polar spot on TLC thus it was advanced further without purification or characterization. The crude product was reacted with 2,6-diaminopyrimidin-4(3H)-one (26d) and sodium acetate at 45 °C for 4 h. No cyclized product, ethyl 5-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinate (27d), was formed. Alternatively, reaction of 22d with 5,5-dibromo-2,2-dimethyl-1,3-dioxane-4,6-dione (24d) in 1N HCl in diethyl ether (condition B) resulted in a more polar spot (not observed with Br$_2$/dioxane, condition A) on TLC and was taken advanced to the next step without purification. The reaction of the crude product from condition B with 26d resulted in a pink spot on TLC, which upon chromatography purification was confirmed as the cyclized product 27d. Hydrolysis of the pteroic ester resulted in acid 28d. Glutamate coupling (65%) and saponification afforded (5-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinoyl)-L-glutamic acid (14c) in 78% yield as a white solid.

**Scheme 50:** Synthesis of intermediate 30d.

To synthesize other compounds in the series (13c, 15c and 16c), 5-bromopicolinic acid (20d) (Scheme 50) was first reacted with diethyl L-glutamate to synthesize the key intermediate diethyl (5-bromopicolinoyl)-L-glutamate (30d). Using 30d and the alternative synthetic scheme (using Sonogashira coupling and 5,5-dibromo-2,2-
dimethyl-1,3-dioxane-4,6-dione (24d) for bromination) was utilized to synthesize target compounds (Scheme 51).

**Scheme 51:** Synthesis of target compounds 13c, 15c and 16c.

The key intermediate aldehydes (37d-39d) (Scheme 51) were synthesized using the optimized procedure from Scheme 48 through the Sonogashira coupling, hydrogenation and
oxidation. The $\alpha$-bromination of the aldehydes and the cyclization reaction of the corresponding $\alpha$-bromo aldehydes (40d-42d) with 2,6-diaminopyrimidin-4(3H)-one (26d) afforded the pteroic esters (43d-45d) in 25-40% yield over two steps. Saponification of the pterooid esters (43d-45d) gave the target compounds (13c, 15c and 16c) in 63-75%.

**Scheme 52:** Synthesis of target compounds 17c, 18c and 19c.
The target compounds 17c-19c were synthesized using the synthetic procedure presented in Scheme 52. Glutamate coupling was performed on the 6-bromonicotinic acid (46d) to afford diethyl (6-bromonicotinoyl)-L-glutamate (47d) in 83% yield as a clear oil. Sonogashira coupling, catalytic reduction and oxidation of the corresponding alcohols (51d-53d) afforded the aldehydes (54d-56d) in 47-60% yield. The α-bromination, cyclization and hydrolysis resulted in the final acids 17c-19c in 64-75% yield. The longer time (upto 2 h) taken for complete D$_2$O exchange of the amide-NH of 13c -16c in comparison to the regioisomers 17c-19c (D$_2$O exchange time about 10 min) provided partial proof for possible intra-molecular H-bonding in 13c-16c.$^{349,350}$

B.2. Synthesis of dual inhibitors of cytosolic (de novo purine biosynthetic) and mitochondrial (serine hydroxymethyltransferase 2) IC metabolism enzymes

1. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-d]pyrimidine-pyridyl classical antifolates

The proposed synthetic scheme for the target compounds **20c-22c** involved synthesis of pteroic acids (**63d-65d**) (**Scheme 53**). The key step for the pteroic acids synthesis was alkylation of the pyrrole nitrogen of 2-amino-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (**66d**) using methyl 5-(3-iodoalkyl)picolinate (**67d-69d**).

**Scheme 54**: Synthesis of the key intermediates methyl 5-(3-iodoalkyl)picolinates (**67d-69d**).

Sonogashira coupling of corresponding alkyn-1-ol with methyl 5-bromopicolinate (**70d**) (**Scheme 54**) using previously reported conditions afforded methyl 5-(3-hydroxyalk-1-yn-1-yl)picolinates (**71d-73d**) as clear oils in 63-70% yield. Subsequently, catalytic hydrogenation afforded methyl 5-(3-hydroxyalkyl)picolinates (**74d-76d**) in 75-88% yield. To make the hydroxyl groups of **74d-76d** better leaving groups it was functionalized into a mesyl group using mesyl chloride to afford **77d-79d** in 64-70% yield. Subsequently, a Finkelstein reaction was carried out to obtain the methyl 5-(3-iodoalkyl)picolinate (**67d-69d**) in 64-70% yield. This two-step synthetic scheme afforded iodides (**67d-69d**) in 37-50% yield over two steps from alcohols **74d-76d**. Alternatively, a one pot reaction from alkyl alcohols (**74d-76d**)
using triphenylphosphine, imidazole and iodine afforded iodides (67d-69d) in 80-85% yield.

This method was a significant improvement over the previous two-step method for conversion of the alcohol to mesylate and to the iodide (Scheme 54). This procedure requires one step, one workup and one column chromatographic separation and is more efficient, compared to the previous method (Scheme 54), which needs two steps, two workups and a column chromatographic separation. It is also less time consuming.

**Scheme 55:** Synthesis of the key intermediate 2-amino-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (66d).

To increase the solubility of the pyrrolo[3,2-d]pyrimidine (66d) (Scheme 55) for N-alkylation, the pivaloyl protected derivative of 66d (84d) was synthesized using the procedure in Scheme 55. Nitration at the 5-position of 2-amino-6-methylpyrimidin-4(3H)-one (80d) afforded 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (81d) in 97% yield as a yellow solid. Treatment of 81d with N,N-dimethylformamide dimethyl acetal in DMF afforded N'-(4-(2-(dimethylamino)vinyl)-5-nitro-6-oxo-1,6-dihydropyrimidin-2-yl)-N,N-dimethylformimidamide (82d) in 90% yield. Reductive cyclization of 82d with sodium
dithionite in water/THF under reflux yielded $N,N$-dimethyl-$N'$-(4-oxo-4,5-dihydro-$3H$-pyrrolo[3,2-$d$]pyrimidin-2-yl)formimidamide ($83d$) in 89% yield. Hydrolysis and pivaloylation of $83d$ afforded the protected derivative of $66d$ ($84d$) as a white solid.\textsuperscript{351}

**Scheme 56**: Attempted $N$-alkylation of $84d$.

![Diagram showing the reaction between $84d$ and $67d$](image)

The crucial $N$-alkylation step was first attempted with sodium hydride (Scheme 56) as a base. Instead of mono-alkylation product ($85d$) (Scheme 56) the reaction condition caused alkylation at both the 3-NH (lactam) and the 5-NH (pyrrole) positions of $84d$ to give methyl 5-(3-(5-(3-(4-(methoxycarbonyl)phenyl)propyl)-4-oxo-2-pivalamido-4,5-dihydro-$3H$-pyrrolo[3,2-$d$]pyrimidin-3-yl)propyl)picolinate ($86d$). Alternatively, using a weaker base such as cesium carbonate resulted in only 10% yield with most of the starting material, $84d$, recovered unreacted. Therefore, an alternative procedure\textsuperscript{254} was adopted which involved protecting both the 2-NH$_2$ and 3-NH group of the pyrrolo[3,2-$d$]pyrimidine to make the $N$-alkylation cleaner and increase the yield (Scheme 57).

Treatment of DMF dimethylacetal with $81d$ (Scheme 57) in dichloromethane gave protection at the 2-NH$_2$ position to give $N,N$-dimethyl-$N'$-(4-methyl-5-nitro-6-oxo-1,6-dihydropyrimidin-2-yl)formimidamide ($87d$) in 60% yield over 4 h. The resulting brown oil ($88d$) was reacted with excess of chloromethyl pivalate and 1.1 eq. of sodium hydride to give
a mixture of N3-alkylated (89d, 65%) and O4-alkylated products (90d, 15%) which were separated by column chromatography and characterized by $^{13}$C NMR (see Experimental section). Next step involved reacting 89d or 90d with DMF dimethylacetal. Reaction of O-alkylated 90d with DMF dimethylacetal resulted in incomplete conversion and a mixture of products. N-alkylated 89d however gave full conversion and a clean reaction to afford (2-((dimethylamino)methylene)amino)-4-(2-(dimethylamino)vinyl)-5-nitro-6-oxopyrimidin-1(6H)-yl)methyl pivalate (91d) in 90% yield as a yellow powder. Subsequent reduction of the nitro group of 91d with sodium hydrosulfite and simultaneous intramolecular cyclization resulted in (2-(((dimethylamino)methylene)amino)-4-oxo-5,4-dihydro-3H-pyrrolo[3,2-d]pyrimidin-3-yl)methyl pivalate (92d) in 89% yield.

**Scheme 57:** Synthesis of the intermediate 92d.
Scheme 58: Synthesis of target compounds (20c-22c).

The N-alkylation at the pyrrole nitrogen (5-position) of 92d (Scheme 58) with the corresponding alkyl iodides (67d-69d) gave the 5-substituted pyrrolo[3,2-\textit{d}]pyrimidines (93d-95d) in moderate yields (40-50\%). This method improved yields and gave cleaner reactions compared to the previous N-alkylation procedure (Scheme 56). Simultaneous deprotection and hydrolysis of the esters, subsequent glutamate coupling and saponification afforded target compounds 20c-22c in 74-90\% yield. D$_2$O study indicated that possible intramolecular H-bonding of amide NH with pyridyl N may be responsible for increased time (> 2 h vs ~ 10 min) taken for complete D$_2$O exchange of amide NH.\textsuperscript{350}

2. 5-Substituted, 2-amino-4-oxo pyrrolo[3,2-\textit{d}]pyrimidine-3'-fluoropyridyl classical antifolates

The standard amide coupling reagent, 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), used for the synthesis of previous series of target compounds gave ~20\% yield for the reaction
of 5-bromo-3-fluoropicolinic acid (102d) (Scheme 59) and diethyl L-glutamate. Alternatively, using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)\textsuperscript{350} improved the amide coupling reaction yield to 90% to afford the diethyl (3-fluoro-5-bromopicolinoyl)-L-glutamate (103d) as a clear oil (Scheme 59). The glutamate 103d was Sonogashira coupled with the alkyn-1-ols to afford diethyl (3-fluoro-5-(hydroxyalk-1-yn-1-yl)picolinoyl)-L-glutamates (104d-105d) in 65-69% yield as yellow oils. Subsequent catalytic hydrogenation, mesylation and Finkelstein reaction afforded diethyl (3-fluoro-5-(iodoalkyl)picolinoyl)-L-glutamates (110d-111d) in excellent yields.

Scheme 59: Synthesis of intermediate diethyl (3-fluoro-5-(iodoalkyl)picolinoyl)-L-glutamates (110d-111d)
Scheme 60: Synthesis of target compounds 23c-24c.

N-alkylation, deprotection and hydrolysis afforded target compounds 23c-24c (Scheme 60) as white solids.

B.3. Synthesis of 5-substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-d]/[3,2-d]pyrimidine-pyridyl classical antifolates

Scheme 61: Retrosynthesis of target compound 25c.

The synthesis of the 5-substituted, 2-amino-4-oxo-6-methyl pyrrolo[2,3-d]pyrimidine pyridyl target compound 25c (Scheme 61) was envisioned via a Fischer indole cyclization of
the hydrazine 115d and ketone 116d. The ketone, methyl 5-(5-oxohexyl)picolinate (116d), was to be synthesized via a Heck coupling of the 5-bromopicolinate (118d) and 5-hexene-2-one (117d).

**Scheme 62:** Heck coupling for the synthesis of intermediate 116d.

Treatment of 118d (Scheme 62) with 117d using the previously reported Heck coupling conditions\(^\text{185}\) gave a mixture of regioisomers of alkene 119d, indicative of palladium assisted migration of unsaturation along the hydrocarbon chain towards the tethered carbonyl group of the ketone (Scheme 62).\(^\text{352}\) The presence of these different alkene regioisomers were confirmed via \(^1\)H NMR spectrum. The spectrum clearly shows three different sets of peaks for three different types of pyridyl protons of the regioisomers of 119d (Fig. 81). The sets of the protons \(H_a (\delta = 8.67-8.81, \text{d, ortho to the pyridyl } N)\), \(H_b (\delta = 8.07-8.17, \text{d, para to the pyridyl } N)\) and \(H_c (\delta = 7.78-7.96, \text{d, meta to the pyridyl } N)\) in the \(^1\)H NMR indicated the presence of three regioisomers of 119d formed via the Heck coupling reaction. The regioisomers of 119d were taken further without purification for hydrogenation to afford a single product 116d in 80% yield.
Figure 81: NMR spectrum showing mixture of alkene isomers of 119d afforded by the Heck coupling reaction, three sets of proton peaks seen for each pyridine hydrogens (Hₐ, Hₖ and Hₖ) corresponding to three regioisomers
Scheme 63: Attempted synthesis of intermediate 114d using Fischer-indole synthesis.

Condensation of ketone 116d with the hydrazine 115d under reflux in 2-methoxyethanol afforded the key intermediate hydrazonopyrimidine 121d in 83% yield as a brown solid (Scheme 63). However, the Fischer indole condensation of 121d in diphenyl ether at 250 °C led to charred material without yielding the desired cyclized product 114d. The failure of this condensation was attributed to the low melting point (~183 °C) of 114d which decomposed at the high temperature conditions of the Fischer indole condensation.

Scheme 64: Synthesis of intermediate ethyl 3-amino-5-methyl-1/H-pyrrole-2-carboxylate (125d)
Using a literature method,\textsuperscript{354} the synthesis of ethyl 3-amino-5-methyl-1\textit{H}-pyrrole-2-carboxylate, \textit{125d} was accomplished (Scheme 64). The crucial intermediate \textit{124d} (E/Z mixture) was readily obtained from 3-aminobut-2-enenitrile \textit{122d} (E/Z-mixture) and diethyl aminomalonate hydrochloride, \textit{123d}, in methanol at room temperature overnight. The TLC shows multiple spots and the reaction required extensive chromatographic separation. The formation of a light pink spot on TLC is an indication of the desired intermediate \textit{124d}, and it was advanced to the cyclization along with impurities. The intermediate diethyl 2-((1-cyanoprop-1-en-2-yl)amino)malonate \textit{124d} under reflux conditions with sodium ethoxide in ethanol afforded intermediate \textit{125d} in 60\% yield over two steps.

The N-alkylation of ethyl 5-methyl-3-amino-1\textit{H}-pyrrole-2-carboxylate \textit{125d} using iodide \textit{68d} (Scheme 65) and sodium hydride under anhydrous conditions afforded the \textit{N}-5-substituted pyrrole \textit{126d}.\textsuperscript{354} The crude \textit{N}-substituted pyrrole \textit{126d} was directly subjected to condensation with 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea with 5 equivalent of acetic acid as catalyst and MeOH. Hydrolysis of the carbamate group with aqueous sodium hydroxide at 55 °C afforded the 2-amino-4-oxo-6-methyl-pyrrolo[3,2-\textit{d}]pyrimidine \textit{128d} in 25\% yield (over three steps). Conversion of the free acid \textit{128d} to the corresponding L-glutamic acid diethyl ester \textit{129d} involved conventional peptide coupling with L-glutamic acid diethyl ester hydrochloride using 2-chloro-4,6-dimethoxy-1,3,5-triazine followed by chromatographic purification to afford the coupled products in 64\% yields. Hydrolysis of \textit{129d} with aqueous NaOH at room temperature, followed by acidification with 1 N HCl, afforded target compound \textit{26c} in 73\% yield.
Scheme 65: Synthesis of target compound 26c.
C. Synthesis of selective inhibition of *P. jirovecii* dihydrofolate reductase

1. 5-substituted, 2,4-diamino pyrimidines (trimethoprim analogs) as selective pjDHFR inhibitors

**Scheme 66:** Synthesis of the target compounds 28c-31c.

Protection of 2,4-dichloropyrimidine (130d) (Scheme 66) with the tert-butoxy group provided 5-bromo-2,4-di-tert-butoxypyrimidine (131d) in 87% yield.\(^{355, 356}\) Treatment of 5-bromo-2,4-di-tert-butoxypyrimidine (131d) with iPrMgClLiCl (0 °C, 4 h) provided the intermediate magnesium reagent in >95% yield.\(^{357}\) Its cross-coupling with the corresponding diethyl benzyl phosphates (132d-136d) (60 °C, 1 h) led to 2,4-di-tert-butoxy-5-substituted-pyrimidines (137d-141d) in 40-60% yield. The pyrimidine derivatives 137d-141d were deprotected using concentrated HCl in methanol to give the corresponding uracil derivatives, which were converted to the 2,4-dichloro-5-substituted pyrimidines (142d-146d) with POCl\(_3\).\(^{358}\) The nucleophilic substitution of the chlorines by two amino functions was performed by heating the 2,4-dichloro pyrimidine derivatives 142d-146d with a 7 M solution of ammonia.
in methanol at 150 °C in a microwave reactor, providing the desired trimethoprim analogs
(28c-32c) in 30-40% yield over three steps (Scheme 66).
V. EXPERIMENTAL

All evaporations were carried out in vacuum with a rotary evaporator. 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 Sorbetch silica g TLC plates 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 prepacked RediSep® Rf normal-phase flash columns (230 to 400 meshes) of various sizes were used. 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 systems and were analyzed using MestReC NMR data processing software. The chemical shift values (δ) are expressed in ppm (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. 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 ¹H NMR spectra. Mass spectrum data were acquired on an Advion expressionS CMS system using the ESI probe. All
solvents and chemicals were purchased from Sigma-Aldrich or Fisher Scientific and were used as received.

*N*-Methyl-4-(methylthio)aniline (2d).

To a solution of sodium methoxide (1.94 g, 36 mmol) in 25 mL of MeOH in a 100 mL round bottom flask was added 4-(methylthio)aniline 1d (0.89 ml, 7.18 mmol). The resultant solution was then added to a 100 mL round-bottomed flask containing paraformaldehyde (0.3 g, 10.06 mmol) in 25 mL of MeOH. The reaction mixture was stirred at rt under argon atmosphere. After 5 h, sodium borohydride (0.27 g, 7.18 mmol) was added, and the reaction mixture was heated to reflux for 2 h. After cooling to rt, silica gel (3 g) was added, and the solvent was removed in vacuo. The resultant plug was chromatographed and eluted with 5% EtOAc/hexane. Fractions containing the product (TLC) were pooled and evaporated to afford 2d as a light brown liquid (0.98 g, 89%). TLC $R_f = 0.76$ (hexane/EtOAc, 1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.33$ (s, 3H, SCH$_3$), 2.64–2.66 (s, 3H, NCH$_3$), 5.72–5.73 (d, $J = 7.0$ Hz, 1H, NH), 6.50–6.52 (d, $J = 6.8$ Hz, 1H, Ar), 7.11–7.13 (d, 2H, Ar). $^1$H NMR agreed well with the literature reported values. Mass calculated for C$_8$H$_{11}$NS [M + H]$^+$, 154.06; Found 154.06.

*Tert-*butyl (5-hydroxynaphthalen-2-yl)carbamate (5d).

To a 100 mL round-bottomed flask was added 6-aminonaphthalen-1-ol 4d (2 g, 12.56 mmol), DMAP (0.15 g, 1.26 mmol), triethylamine (1.75 mL, 12.56 mmol) and 1M solution of di-*tert-*butyl dicarbonate in THF (41.46 mL) and stirred overnight at rt. TLC showed completion of the reaction. The crude mixture was chromatographed using 10% EtOAc/Hexanes and fractions containing the product were pooled to give 2.8 g of 5d (86%) as a black liquid. TLC $R_f = 0.63$ (Hexanes/EtOAc, 3:1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.80 (dt, $J = 8.5, 1.0$ Hz, 1H, Ar), 7.49 (dt, $J = 8.3, 1.1$ Hz, 1H, Ar), 7.36 (dd, $J = 8.2, 7.5$ Hz, 1H, Ar), 7.07 (dd, $J = 7.6, 1.1$ Hz, 1H, Ar), 7.04 – 6.97 (m, 2H, Ar), 1.61 (s, 9H, CH$_3$).
Tert-butyl (5-methoxynaphthalen-2-yl)(methyl)carbamate (6d).

A 250 mL round-bottomed flask was equipped with a stir bar and charged with 5d (2.8 g, 10.80 mmol) and an anhydrous DMF (100 mL) and cooled in ice bath. To this was added 60% dispersion of NaH in oil (1.3 g, 32.39 mmol) under argon and kept at 0 °C for 15 min. After 15 min, methyl iodide (1.48 mL, 23.76 mmol) was added dropwise to the solution and reaction was warmed to rt. After 2 h, silica gel was added and DMF was evaporated in vacuo to make plug. The plug was purified using column chromatography, eluting with 10% EtOAc/Hexanes to afford 2.79 g of 6d (90%) as a brown liquid. TLC R$_f$ = 0.90 (Hexanes/EtOAc, 1:1) $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.19 (d, J = 9.0 Hz, 1H, Ar), 7.58 (d, J = 2.2 Hz, 1H, Ar), 7.42 – 7.33 (m, 3H, Ar), 6.78 (dd, J = 6.1, 2.5 Hz, 1H, Ar), 4.00 (s, 3H, OCH$_3$), 3.36 (s, 3H, NCH$_3$), 1.46 (s, 9H, CH$_3$). Mass calculated for C$_{17}$H$_{21}$NO$_3$ [M + H]$^+$, 288.16; Found 288.15.

5-Methoxy-N-methylnaphthalen-2-amine (7d).

In a 100 mL round-bottomed flask was added 6d (2.79 g, 9.71 mmol) and trifluoroacetic acid (30 mL) and stirred for 2 h at rt. The reaction mixture was chromatographed, eluting with 10% EtOAc/Hexanes to afford 1.70 g of 7d (93%) as a black semisolid. TLC R$_f$ = 0.78 (Hexanes/EtOAc, 1:1). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 8.06 (d, J = 9.1 Hz, 1H, Ar), 7.35 – 7.22 (m, 2H, Ar), 6.88 (ddd, J = 9.0, 2.4, 0.9 Hz, 1H, Ar), 6.79 (d, J = 2.3 Hz, 1H, Ar), 6.60 (dd, J = 7.3, 1.2 Hz, 1H, Ar), 3.99 (s, 3H, OCH$_3$), 2.96 (s, 3H, NCH$_3$). Mass calculated for C$_{12}$H$_{23}$NO [M + H]$^+$, 188.10; Found 188.10.

4-Chloroquinazoline (9d).

To a 250 mL round-bottomed flask were added quinazolin-4(3H)-one 8d (1.11 g, 7.59 mmol), trimethylamine (1.36 mL, 9.87 mmol), and toluene (20 mL). The mixture was cooled to 0 °C to which phosphorus oxychloride (0.85 mL, 9.11 mmol) was added and stirred at room temperature for 1 h. The reaction was heated to 95 °C for 3 h. After monitoring the reaction on TLC, the reaction was cooled to room temperature and diluted with 30 mL of ethyl acetate.
The solution was then washed with 10 mL of ice-cold water, 10 mL of saturated NaHCO₃, 10 mL of water, 1N HCl (5 mL), 10 mL of water, 10 mL of saturated NaHCO₃ and 10 mL of saturated NaCl. The organic layer was dried over Na₂SO₄, filtered and concentrated to obtain 9d as white solid (1.08 g, 80%). TLC Rₛ = 0.85 (MeOH:CHCl₃; 1:5); mp, 96.5 – 97.5 °C (lit.³⁶⁰ 97 - 98 °C); ¹H NMR, DMSO-d₆ (400 MHz): 7.59-7.63 (dt, 1 H, J₁ = 1.09, J₂ = 7.23, J₃ = 8.01, Ar), 7.76-7.78 (d, 1 H, J = 8.24, Ar), 7.89-7.92 (dt, 1 H, J₁ = 1.47, J₂ = 7.24, J₃ = 8.29, Ar), 8.15-8.17 (d, 1 H, J = 7.03, Ar), 8.58 (s, 1 H, Ar).


In a 100 mL round-bottomed flask, 3-aminopicolinic acid 10d (4 g, 28.96 mmol) and urea (17.39 g, 289.59 mmol) were mixed and kept in a preheated oil bath at 160 °C overnight, then 50 mL of water was added and mixture was cooled down to rt and filtered. The crude was dissolved in 0.2 M NaOH stirred again at 100 °C for 20 min and cooled down to rt. The solution was filtered and washed with water/MeOH (1/1 v/v) and dried to afford 3.78 g (80%) of 11d as a white solid. mp, >300 °C (lit.³⁶¹ 320 °C); ¹H NMR (400 MHz, DMSO-d₆) δ 11.50 (s, 1H, NH), 11.23 (s, 1H, NH), 8.46 (d, J = 4.1 Hz, 1H, Ar), 7.62 (d, J = 4.3 Hz, 1H, Ar), 7.57 (d, J = 8.1 Hz, 1H, Ar). Mass calculated for C₇H₅N₃O₂ [M + H]^⁺, 164.04; Found 164.05.

2,4-Dichloropyrido[3,2-β]pyrimidine (12d).

To a 250 mL round-bottomed flask were added pyrido[3,2-β]pyrimidine-2,4(1H,3H)-dione (11d) (2 g, 12.26 mmol), trimethylamine (2.22 mL, 15.94 mmol), and toluene (40 mL). The mixture was cooled to 0 °C to which phosphorus oxychloride (2.51 mL, 26.97 mmol) was added and stirred at room temperature for 1 h. The reaction was heated to 95 °C for 3 h. After monitoring the reaction on TLC, the reaction was cooled to room temperature and diluted with 30 mL of ethyl acetate. The solution was then washed with 10 mL of ice-cold water, 10 mL of saturated NaHCO₃, 10 mL of water, 1N HCl (5 mL), 10 mL of water, 10 mL of saturated NaHCO₃ and 10 mL of saturated NaCl. The organic layer was dried over Na₂SO₄, filtered and
concentrated to obtain 12d as white solid (1.68 g, 68%). TLC $R_f = 0.65$ (MeOH:CHCl$_3$; 1:5); mp, 165.5 °C (lit.$^{360}$ 165-167 °C) $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.22 (dd, $J$ = 4.1, 1.5 Hz, 1H, Ar), 8.50 (dd, $J$ = 8.6, 1.5 Hz, 1H, Ar), 8.16 (dd, $J$ = 8.6, 4.1 Hz, 1H, Ar).

Pyrido[3,2-$d$]pyrimidin-4(3$H$)-one (14d)

To a 2-5 mL Biotage® microwave vial were added 3-aminopicolinic acid 10d (1 g, 7.24 mmol) and formamide 13d (2 mL, 50.68 mmol) which were set up for microwave irradiation at 170 °C for 60 min. To this was added 10 mL water and the resulting suspension was filtered and washed with water (10 mL x 2), dried over P$_2$O$_5$ to yield 14d as buff colored solid (0.38 g, 36%). TLC $R_f = 0.38$ (MeOH:CHCl$_3$; 1:5); mp, $>300$ °C (lit.$^{362}$ 346-347 °C); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.56 (s, 1H, NH), 8.79 (dd, $J$ = 4.3, 1.6 Hz, 1H, Ar), 8.16 (s, 1H, Ar), 8.09 (dd, $J$ = 8.4, 1.6 Hz, 1H, Ar), 7.82 (dd, $J$ = 8.3, 4.3 Hz, 1H, Ar).

4-Chloropyrido[3,2-$d$]pyrimidine (15d).

To a 250 mL round-bottomed flask was added pyrido[3,2-$d$]pyrimidin-4(3$H$)-one (14d) (1.3 g, 8.84 mmol), triethylamine (1.6 mL, 11.49 mmol), and toluene (20 mL). The mixture was cooled to 0 °C to which phosphorus oxychloride (0.99 mL, 10.60 mmol) was added and stirred at room temperature for 1 h. The reaction was heated to 95 °C for 3 h. After monitoring the reaction on TLC, the reaction was cooled to room temperature and diluted with 30 mL of ethyl acetate. The solution was then washed with 10 mL of ice-cold water, 10 mL of saturated NaHCO$_3$, 10 mL of water, 1N HCl (5 mL), 10 mL of water, 10 mL of saturated NaHCO$_3$ and 10 mL of saturated NaCl. The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated to obtain 15d as yellow colored solid (1.03 g, 70%). TLC $R_f = 0.78$ (MeOH:CHCl$_3$; 1:5); mp, 140.3 °C decomposed (lit.$^{363}$ 136-139 °C decomposed); $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.24 – 9.18 (m, 1H, Ar), 9.17 (s, 1H, Ar), 8.48 – 8.42 (m, 1H, Ar), 7.95 (d, $J$ = 8.8 Hz, 1H, Ar).

**General procedure for the synthesis of quinazoline (1c and 2c) and pyrido[3,2-$d$]pyrimidine analogs (3c - 10c).**
To a 50 mL round-bottomed flask were added 1 equivalent of 4-chloro compounds (6d, 9d, 13d, and 15d) and 1 or 1.1 equivalent of appropriate anilines, acetonitrile (1c and 2c) or isopropanol (3c-10c) and a drop of conc. HCl and stirred at room temperature for 12 h. The compounds precipitated as a HCl salt, which were filtered and dried in vacuo over P₂O₅. If further purification was required, then the compounds were purified using column chromatography to give yellow solids.

**N-(5-Methoxynaphthalen-2-yl)-N-methylquinazolin-4-amine (1c).**

Using the general procedure described above, the reaction of 6d (0.2 g, 1.22 mmol) and 4d (0.23 g, 1.22 mmol) resulted in 1c (0.33 g, 85%) as yellow solid. TLC Rₓ = 0.85 (MeOH:CHCl₃; 1:5); mp: 123.6 °C, ¹H NMR (400 MHz, Chloroform-d) δ 7.95 (s, 1H, Ar), 7.64 (s, 1H, Ar), 7.32 (d, J = 8.6 Hz, 2H, Ar), 7.17 (d, J = 8.6 Hz, 2H, Ar), 7.10 (s, 1H, Ar), 7.02 – 6.97 (m, 1H, Ar), 3.68 (s, 3H, NCH₃), 2.55 (s, 3H, SCH₃). Anal. Calcd. for C₁₆H₁₄ClN₃S: C, 60.85; H, 4.47; N, 13.31; S, 10.15; Cl, 11.22. Found: C, 60.97; H, 4.55; N, 13.19; S, 10.12; Cl, 11.07.

**2-Chloro-N-methyl-N-(4-(methylthio)phenyl)quinazolin-4-amine (2c).**

Using the general procedure described above, the reaction of 9d (0.3 g, 1.51 mmol) and 7d (0.23 g, 1.51 mmol) resulted in 2c (0.1 g, 22%) as dark yellow solid. TLC Rₓ = 0.76 (MeOH:CHCl₃; 1:5); mp, 243 °C, ¹H NMR (400 MHz, Chloroform-d) δ ¹H NMR (400 MHz, Chloroform-d) δ 7.95 (d, J = 8.3 Hz, 1H, Ar), 7.64 (d, J = 8.4, 6.9, 1.4 Hz, 1H, Ar), 7.37 – 7.30 (m, 2H, Ar), 7.20 – 7.14 (m, 2H, Ar), 7.10 (d, J = 8.4, 7.0, 1.4 Hz, 1H, Ar), 6.99 (dd, J = 8.7, 1.4 Hz, 1H, Ar), 3.68 (s, 3H, NCH₃), 2.55 (s, 3H, NCH₃). Anal. Calcd. for C₂₀H₁₇N₃O₁.09 HCl: C, 67.61; H, 5.13; N, 11.82; Cl, 9.41. Found: C, 67.61; H, 5.26; N, 11.85; Cl, 9.89.

**2-Chloro-N-(4-methoxyphenyl)-N-methylpyrido[3,2-d]pyrimidin-4-amine (3c).**

Using the general procedure described above, the reaction of 12d (0.06 g, 0.36 mmol) and 4-methoxy-N-methylaniline (0.06 g, 0.4 mmol) resulted in 3c (0.09 g, 93%) as yellow solid. TLC Rₓ = 0.83 (MeOH:CHCl₃; 1:5); mp: 245.1-248.3 °C, ¹H NMR (400 MHz, Chloroform-d) δ 9.16
(dd, J = 4.4, 1.8 Hz, 1H, Ar), 8.39 (dd, J = 8.4, 1.7 Hz, 1H, Ar), 7.90 (dd, J = 8.7, 4.1 Hz, 1H, Ar), 7.33 (d, J = 8.3 Hz, 2H, Ar), 7.15 (d, J = 8.4 Hz, 2H, Ar), 3.83 (s, 3H, OCH$_3$), 2.56 (s, 3H, NCH$_3$). Anal. Calcd. for C$_{15}$H$_{13}$ClN$_4$O 0.18 CH$_3$OH 0.24 HCl: C, 57.79; H, 4.46; N, 17.75; Cl, 13.96. Found: C, 58.06; H, 4.65; N, 17.43; Cl, 14.08.

4-(6-Methoxy-3,4-dihydroquinolin-1(2H)-yl)pyrido[3,2-d]pyrimidine (4e).

Using the general procedure described above, the reaction of 12d (0.18 g, 1.09 mmol) and 6-methoxy-1,2,3,4-tetrahydroquinoline (0.18 g, 1.09 mmol) resulted in 4e (0.25 g, 79%) as yellow solid. TLC $R_f$ = 0.89 (MeOH:CHCl$_3$; 1:5); mp: 139.8 °C (lit.$^{364}$ 137-138 °C), $^1$H NMR (400 MHz, DMSO-$_d_6$) $\delta$ 8.69 (dd, J = 4.1, 1.6 Hz, 1H, Ar), 8.11 (dd, J = 8.5, 1.7 Hz, 1H, Ar), 7.81 (dd, J = 8.6, 4.2 Hz, 1H, Ar), 7.09 (d, J = 8.9 Hz, 1H, Ar), 6.80 (d, J = 3.0 Hz, 1H, Ar), 6.65 (dd, J = 8.9, 3.0 Hz, 1H, Ar), 4.33 (t, J = 6.3 Hz, 2H, CH$_2$), 3.76 (d, J = 1.7 Hz, 3H, OCH$_3$), 2.81 (dt, J = 6.7 Hz, 2H, CH$_2$). Anal. Calcd. for C$_{17}$H$_{15}$ClN$_4$O 0.218 CH$_3$COCH$_3$: C, 62.46; H, 4.84; N, 16.50; Cl, 10.44. Found: C, 62.67; H, 4.85; N, 16.73; Cl, 10.06.

2-Chloro-N-methyl-N-(4-(methylthio)phenyl)pyrido[3,2-d]pyrimidin-4-amine (5c).

Using the general procedure described above, the reaction of 12d (0.15 g, 0.9 mmol) and N-methyl-4-(methylthio)aniline (0.15 g, 0.99 mmol) resulted in 5c (0.1 g, 39%) as yellow solid. TLC $R_f$ = 0.84 (MeOH:CHCl$_3$; 1:5); mp: 251.4 °C, $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 9.18 (dd, J = 4.2, 1.6 Hz, 1H, Ar), 8.37 (dd, J = 8.5, 1.6 Hz, 1H, Ar), 7.95 (dd, J = 8.7, 4.2 Hz, 1H, Ar), 7.31 (d, J = 8.3 Hz, 2H, Ar), 7.14 (d, J = 8.4 Hz, 2H, Ar), 3.86 (s, 3H, OCH$_3$), 2.58 (s, 3H, NCH$_3$). Anal. Calcd. for C$_{15}$H$_{13}$ClN$_4$S: C, 56.87; H, 4.14; N, 17.69; Cl, 11.19; S, 10.12 Found: C, 56.80; H, 4.48; N, 17.44; Cl, 11.40; S, 9.78.

2-Chloro-N-(5-methoxynaphthalen-2-yl)-N-methylpyrido[3,2-d]pyrimidin-4-amine (6c).

Using the general procedure described above, the reaction of 12d (0.2 g, 1.21 mmol) and 5-methoxy-N-methylnaphthalen-2-amine (0.23 g, 1.21 mmol) resulted in 6c (0.25 g, 65%) as
yellow solid. TLC $R_f = 0.82$ (MeOH:CHCl$_3$; 1:5); mp: 187.2 °C, $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.31 (dd, $J = 4.1$, 1.6 Hz, 1H, Ar), 8.11 (d, $J = 8.9$ Hz, 1H, Ar), 8.06 (dt, $J = 8.5$, 4.2 Hz, 1H, Ar), 7.83 (d, $J = 2.2$ Hz, 1H, Ar), 7.71 – 7.63 (m, 1H, Ar), 7.48 – 7.37 (m, 3H, Ar), 7.01 – 6.96 (m, 1H, Ar), 4.01 (s, 3H, OCH$_3$), 3.78 (s, 3H, NCH$_3$). Anal. Calcd. for C$_{19}$H$_{15}$ClN$_4$O: C, 65.05; H, 4.31; Cl, 10.11; N, 15.87. Found: C, 65.13; H, 4.15; Cl, 10.01; N, 15.64.

$N$-(4-Methoxyphenyl)-$N$-methylpyrido[3,2-d]pyrimidin-4-amine (7c).

Using the general procedure described above, the reaction of 15d (0.11 g, 0.53 mmol) and 4-methoxy-$N$-methylaniline (0.08 g, 0.58 mmol) resulted in 7c (0.12 g, 75.3%) as yellow solid. TLC $R_f = 0.86$ (MeOH:CHCl$_3$; 1:5); mp: 250.4 °C, $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.02 (s, 1H, Ar), 8.66 (s, 1H, Ar), 8.23 (dd, $J = 8.5$, 4.1 Hz, 1H, Ar), 7.88 (dd, $J = 8.5$, 4.2 Hz, 1H, Ar), 7.16 (s, 2H, Ar), 6.98 (s, 2H, Ar), 3.90 (s, 3H, OCH$_3$), 3.86 (s, 3H, NCH$_3$). Anal. Calcd. for C$_{15}$H$_{14}$N$_4$O · 1.01 HCl: C, 59.44; H, 4.99; N, 18.48; Cl, 11.83. Found: C, 59.08; H, 5.14; N, 18.25; Cl, 11.57.

4-(6-Methoxy-3,4-dihydroquinolin-1(2H)-yl)pyrido[3,2-d]pyrimidine (8c).

Using the general procedure described above, the reaction of 15d (0.2 g, 1 mmol) and 6-methoxy-1,2,3,4-tetrahydroquinoline (0.16 g, 1 mmol) resulted in 8c (0.28 g, 85%) as yellow solid. TLC $R_f = 0.86$ (MeOH:CHCl$_3$; 1:5); mp: 237.7 °C, $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.83 (s, 1H, Ar), 8.80 (s, 1H, Ar), 8.24 (dd, $J = 8.6$, 4.3 Hz, 1H, Ar), 7.94 (dd, $J = 8.6$, 4.2 Hz, 1H, Ar), 7.17 (d, $J = 9.0$ Hz, 1H, Ar), 6.85 (d, $J = 2.9$ Hz, 1H, Ar), 6.68 (dd, $J = 8.9$, 2.9 Hz, 1H, Ar), 4.48 (s, 2H, CH$_2$), 3.77 (s, 3H, OCH$_3$), 2.82 (t, $J = 6.8$ Hz, 2H, CH$_2$), 2.02 (q, $J = 6.6$ Hz, 2H, CH$_2$). Anal. Calcd. for C$_{17}$H$_{16}$N$_4$O · 1.0739 HCl: C, 61.59; H, 5.19; N, 16.91; Cl, 11.49. Found: C, 61.63; H, 5.46; N, 16.73; Cl, 11.05.

$N$-Methyl-$N$-(4-(methylthio)phenyl)pyrido[3,2-d]pyrimidin-4-amine (9c).

Using the general procedure described above, the reaction of 15d (0.09 g, 0.45 mmol) and $N$-methyl-4-(methylthio)aniline (0.076 g, 0.5 mmol) resulted in 9c (0.1 g, 70%) as yellow solid.
TLC $R_f = 0.88$ (MeOH:CHCl$_3$; 1:5); mp: 228.1 °C, $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.90 (s, 1H, Ar), 8.66 (s, 1H, Ar), 8.23 (dd, $J = 8.5$, 4.6 Hz, 1H, Ar), 7.88 (dd, $J = 8.5$, 4.2 Hz, 1H, Ar), 7.33 (s, 4H, Ar), 3.89 (s, 3H, NCH$_3$), 2.53 (s, 3H, SCH$_3$). Anal. Calcd. for C$_{15}$H$_{14}$N$_4$S · 0.9951 HCl: C, 56.54; H, 4.74; N, 17.58; S, 10.06; Cl, 11.08. Found: C, 56.55; H, 4.74; N, 17.55; S, 10.06; Cl, 11.17.

$N$-(5-Methoxynaphthalen-2-yl)-$N$-methylpyrido[3,2-$d$]pyrimidin-4-amine (10c).

Using the general procedure described above, the reaction of 15d (0.4 g, 2 mmol) and 5-methoxy-$N$-methylnaphthalen-2-amine (0.37 g, 2 mmol) resulted in 10c (0.49 g, 71 %) as yellow solid. TLC $R_f = 0.9$ (MeOH:CHCl$_3$; 1:5); mp: 122.8 °C, $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.99 (d, $J = 0.6$ Hz, 1H, Ar), 8.60 – 8.46 (m, 1H, Ar), 8.31 – 8.25 (m, 1H, Ar), 8.22 – 8.16 (m, 1H, Ar), 7.93 (d, $J = 1.9$ Hz, 1H, Ar), 7.87 (d, $J = 3.7$ Hz, 1H, Ar), 7.53 – 7.41 (m, 3H, Ar), 7.04 (dd, $J = 7.1$, 0.5 Hz, 1H, Ar), 4.01 (s, 6H, NCH$_3$ and OCH$_3$). Anal. Calcd. for C$_{19}$H$_{16}$N$_4$O: C, 72.13; H, 5.10; N, 17.71. Found: C, 71.93; H, 5.19; N, 17.54.

2-Amino-4-bromo-$1H$-indole-3-carbonitrile (18d).

To an ice-cold solution of malononitrile (4.76 g, 72 mmol) in anhydrous DMF (500 mL) under argon atmosphere was added sodium hydride (2.6 g, 104 mmol). Thus formed white suspension was stirred for 15 min and then 1-bromo-2-fluoro-3-nitrobenzene 16d$^{365}$ (10 g, 36 mmol) was added. Orange color was observed within 2-3 minutes after adding 16d. The mixture was stirred at 110 °C for overnight. After cooling the reaction mixture, 200 mL H$_2$O was added, and the resulting aqueous mixture was acidified to pH 2.0 with conc. HCl. The mixture was extracted with diethyl ether (500 mL × 3) and then the combined organic phases were dried using anhydrous sodium sulfate and concentrated to give (2-Bromo-6-nitrophenyl)-malononitrile 17d as dark-brown oil. TLC $R_f = 0.42$ (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ = 8.19–8.21 (m, 1H, Ar), 8.07–8.09 (m, 1H, Ar), 7.62–7.66 (m, 1H, Ar), 6.18 (s, 1H, CH). The material was used directly for the next step.
A solution of 17d (3.92 g, 14.6 mmol) in glacial acetic acid (500 mL) was treated with a single charge of zinc dust (6.84 g, 104.62 mmol). The mixture was stirred at 55 °C for 45 min, then recharged with zinc dust (3.92 g, 59.96 mmol). After heating for another 105 min, the mixture was filtered through a pad of Celite®. The pad was washed with glacial acetic acid, the filtrate was concentrated and then washed with saturated Na₂CO₃ solution. The precipitate was collected and then dissolved in methanol. Silica gel (5 g) was added to make the plug and was then purified by column chromatography, eluting sequentially with 0% and 1% MeOH in CHCl₃. The fractions containing the pure product (TLC) were pooled and evaporated to give 2.52 g of 18d as a pink solid. The overall yield from 16d to 18d was 60%. TLC Rf = 0.25 (CHCl₃/MeOH, 10:1 with 2 drops of conc. NH₄OH); mp 231.5 °C (lit: 231.1–231.9 °C). ¹H NMR (400 MHz, DMSO-d₆) δ = 10.98 (s, 1H, NH), 7.10–7.12 (m, 1H, Ar), 7.07–7.08 (m, 1H, Ar), 6.89 (bs, 2H, NH₂), 6.78–6.82 (m, 1H, Ar).

5-Bromo-9H-pyrimido[4,5-b]indole-2,4-diamine (19d).

A mixture of 2-amino-4-bromo-1H-indole-3-carbonitrile 18d (3 g, 12.7 mmol), carbamimidic chloride hydrochloride (1.6 g, 13.9 mmol) and methyl sulfone (30 g) was premixed and charged to 100 mL round-bottomed flask. It was transferred to preheated oil bath at 120 °C and reacted for 8 h. The reaction mixture was cooled and 50 mL of 7N ammonia in methanol was added to neutralize the reaction mixture. Solvents were evaporated and the crude mixture was purified by column chromatography, eluting sequentially with 1% and 5% methanol in chloroform. Fractions containing the desired product (TLC) were pooled and evaporated to afford 2.16 g (61%) of 19d as an buff colored solid. TLC Rf = 0.27 (CHCl₃/MeOH, 10:1 with 2 drops of conc. NH₄OH); mp 242 °C (lit: 240 °C). ¹H NMR (400 MHz, DMSO-d₆) δ = 11.49 (br, 1H, 9-NH), 7.24–7.26 (m, 2H, Ar), 7.01–7.05 (m, 1H, Ar), 6.95 (br, 2H, 4-NH₂), 6.05 (bs, 2H, 2-NH₂).
General procedure for the synthesis of 5-(substituted aryloxy)-9H-pyrimido[4,5-b]indole-2,4-diamines 11c-12c.

Compound 19d (1 equivalent), appropriate sodium phenoxides (1 equivalent), copper iodide (2 equivalents), cesium carbonate (2 equivalents) and ligands (1 equivalent) were added to a Biotage® microwave vial. DMF (10 mL) was added as the solvent and the vial was sealed. The reaction was run in a Biotage® Initiator at 180 °C for 1 h. After cooling to room temperature, the reaction mixture was filtered over Celite® pad and washed the pad with 20 mL of DMF. The DMF filtrate was evaporated to concentration (2 mL) and transferred on top of the silica column and purified by column chromatography, sequentially eluting with 1%, 2% and 3% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford the product.

5-Phenoxy-9H-pyrimido[4,5-b]indole-2,4-diamine (11c).

Using the general procedure described above, the reaction of 19d (0.2 g, 0.72 mmol) with sodium phenoxide (0.08 g, 0.72 mmol), copper iodide (0.27 g, 1.44 mmol), cesium carbonate (0.2 g, 1.44 mmol) and 2-aminopyridine 24d (0.068 g, 0.72 mmol) for 1 h afforded 0.15 g (70%) of 11c as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_3$OH); mp >250 °C (decomp), $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 11.33 (s, 1H, NH), 7.43 (t, J = 7.7 Hz, 2H, Ar), 7.20 (t, J = 7.5 Hz, 1H, Ar), 7.15 – 7.10 (m, 2H, Ar), 7.10 – 7.03 (m, 2H, Ar), 6.61 (s, 2H, 4NH$_2$), 6.48 (dd, J = 6.6, 2.5 Hz, 1H, Ar), 5.99 (s, 2H, 2NH$_2$). Anal. Caled. for C$_{16}$H$_{13}$N$_5$O 0.6 CH$_3$OH: C, 64.21; H, 4.99; N, 22.55. Found: C, 64.30; H, 4.68; N, 22.28.

5-(p-Tolyloxy)-9H-pyrimido[4,5-b]indole-2,4-diamine (12c).
Using the general procedure described above, the reaction of 19d (0.2 g, 0.72 mmol) with sodium phenoxide (0.08 g, 0.72 mmol), copper iodide (0.27 g, 1.44 mmol), cesium carbonate (0.2 g, 1.44 mmol) and 2-aminopyridine 24d (0.068 g, 0.72 mmol) for 1 h afforded 0.14 g (68%) of 12c as a dark brown solid. TLC \( R_f = 0.65 \) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp >250 °C (decomp). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 11.15 (s, 1H, NH), 7.41 (t, \( J = 7.7 \) Hz, 2H, Ar), 7.20 (t, \( J = 7.5 \) Hz, 1H, Ar), 7.15 – 7.11 (m, 2H, Ar), 7.10 – 7.01 (m, 2H, Ar), 6.65 (s, 2H, 4-NH\(_2\)), 6.38 (dd, \( J = 6.6, 2.5 \) Hz, 1H, Ar), 5.73 (s, 2H, 2-NH\(_2\)), 2.13 (s, 3H, CH\(_3\)). Anal. Calcd. for C\(_{17}\)H\(_{15}\)N\(_5\)O: C, 66.87; H, 4.95; N, 22.94. Found: C, 66.58; H, 4.70; N, 22.65.

**Ethyl 5-bromopiolinate (21d).**

Thionyl chloride (8.01 g, 67.32 mmol) was added dropwise to a solution of 5-bromopiconic acid (3.4 g, 16.83 mmol) in MeOH (50 mL) and the mixture was stirred overnight at rt.\(^{344}\) After that the mixture was heated to 60 °C for 5 h. The mixture was concentrated, and ethyl acetate was added to the white solid. Saturated NaHCO\(_3\) was added and extracted twice more with ethyl acetate. The combined organic layer was washed with brine, dried over sodium sulfate and evaporated to afford 21d (3.1 g, 80%) as a white solid. TLC \( R_f = 0.70 \) (Hexane/EtOAc, 3:1); Mass calculated for C\(_8\)H\(_8\)BrNO\(_2\) [M + H]\(^+\), 299.97; Found 299.9; \(^1\)H NMR\(^{368}\) (400 MHz, Chloroform-\(d_6\)) \( \delta \) 8.82 (dd, \( J = 2.2, 0.8 \) Hz, 1H, Ar), 8.04 (dd, \( J = 8.4, 0.8 \) Hz, 1H, Ar), 8.00 (dd, \( J = 8.4, 2.2 \) Hz, 1H, Ar), 4.49 (q, \( J = 7.2 \) Hz, 2H, CH\(_2\)), 1.46 (t, \( J = 7.1 \) Hz, 3H, CH\(_3\)).

**Ethyl 5-(5-oxopentyl)picolinate (22d).**

**Condition A:** To a solution of Ethyl 5-bromopiolinate (21d, 1 g, 4.35 mmol) in 20 mL of anhydrous DMF were added pent-4-en-1-ol (1.12 g, 13.04 mmol), LiCl (0.18 g 4.35 mmol), LiOAc (0.72 g, 4.35 mmol), Bu\(_4\)NCl (1.21 g, 4.35 mmol), and Pd(OAc)\(_2\) (0.2 g, 0.87 mmol), and the mixture was stirred at 70 °C for 4 h. TLC (hexane/EtOAc, 3:1) showed the disappearance of the starting material (\( R_f = 0.70 \)) and formation of one major spot at \( R_f = \)
0.50. To the reaction mixture cooled to room temperature was added ethyl acetate (30 mL). The resulting solution was extracted with H$_2$O (10 mL × 3) and dried over Na$_2$SO$_4$. After evaporation of solvent, the residue was loaded on a silica gel column (4 × 20 cm) and flash chromatographed with hexane/EtOAc (2:1), and the desired fractions were pooled. After evaporation of the solvent, the residue was dried in vacuo using P$_2$O$_5$ to afford 22d (95 mg, 10%) as a colorless liquid. R$_f$ = 0.48 (hexane/EtOAc, 3:1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.79 (t, $J$ = 4.1 Hz, 1H, CHO), 8.60 (d, $J$ = 2.3 Hz, 1H, Ar), 8.29 (dd, $J$ = 7.6, 1.7 Hz, 1H, Ar), 7.95 (ddd, $J$ = 8.4, 7.1, 1.6 Hz, 1H, Ar), 7.66 (dd, $J$ = 8.0, 2.3 Hz, 1H, Ar), 4.49 (q, $J$ = 7.1 Hz, 2H, CH$_2$), 2.74 (t, $J$ = 4.2 Hz, 2H, CH$_2$), 2.55 – 2.47 (m, 2H, CH$_2$), 1.75 – 1.67 (m, 4H, CH$_2$), 1.46 (t, $J$ = 7.1 Hz, 3H, CH$_3$). Mass calculated for C$_8$H$_8$BrNO$_2$ [M + K]$^+$, 274.08; Found 274.2; The intermediate 22d was used for the next reaction without further characterization.

**Condition B:** To a solution of ethyl 5-bromopirolinate, 21d (1 g, 4.63 mmol) in anhydrous acetonitrile was added palladium chloride (33 mg, 0.18 mmol), triphenylphosphine (49 mg, 0.18 mmol), copper iodide (141 mg, 0.74 mmol), triethylamine (6.45 mL, 46.3 mmol), and pent-4-yn-1-ol (0.5 mL, 5.6 mmol). The reaction mixture was heated to 100 °C and run for 1 h under microwave heating. A silica plug was prepared by adding silica gel and methanol followed by evaporation of the solvent in vacuo, which was then loaded on to a silica gel column and eluted with hexane followed by 30% EtOAc in hexane. The desired fractions (TLC) were pooled and evaporated to afford the methyl 5-(5-hydroxypent-1-yn-1-yl)picolinate 23d (0.71 g, 70 %) as oil. R$_f$ = 0.40 (hexane/EtOAc, 3:1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.73 (dd, $J$ = 2.1, 0.8 Hz, 1H, Ar), 8.06 (dd, $J$ = 8.2, 0.9 Hz, 1H, Ar), 7.81 (dd, $J$ = 8.1, 2.0 Hz, 1H, Ar), 4.48 (q, $J$ = 7.2 Hz, 2H, CH$_2$), 3.82 (t, $J$ = 6.1 Hz, 2H, CH$_2$), 2.61 (t, $J$ = 7.0 Hz, 2H, CH$_2$), 1.89 (tt, $J$ = 7.1, 6.2 Hz, 2H, CH$_2$), 1.44 (t, $J$ = 7.1 Hz, 3H, CH$_3$). Mass calculated for C$_{13}$H$_{15}$NO$_3$ [M + H]$^+$, 234.11 Found 234.2; The intermediate 23d was used for the next reaction without further characterization.
To 10% palladium on activated carbon (1:1 wt equiv) in a Parr flask, ethanol was added to quench. Methanolic solutions of 23d (2 g, 9.12 mmol) were added, and hydrogenation was carried out at 55 psi of H₂ for 5 h. The reaction mixture was filtered through Celite®, washed with MeOH, passed through a short silica gel column (3 cm × 5 cm), and concentrated under reduced pressure to give methyl 5-(5-hydroxypentyl)picolinate 23d' (2 g, quant. yield) as oil. R_f = 0.38 (hexane/EtOAc, 3:1). ¹H NMR (400 MHz, DMSO-d₆) δ 8.57 (d, J = 2.2 Hz, 1H, Ar), 7.98 (d, J = 8.0 Hz, 1H, Ar), 7.82 (dd, J = 8.0, 2.3 Hz, 1H, Ar), 4.40 – 4.29 (m, 2H, CH₂), 3.38 (td, J = 6.4, 5.1 Hz, 2H, CH₂), 2.68 (t, J = 7.6 Hz, 2H, CH₂), 1.60 (p, J = 7.6 Hz, 2H, CH₂), 1.50 – 1.39 (m, 2H, CH₂), 1.33 (t, J = 7.1 Hz, 3H, CH₃), 1.31 – 1.22 (m, 2H, CH₂). Mass calculated for C₁₃H₁₅NO₃ [M + H]^+ , 238.14 Found 238.3; The intermediate 23d' was used for the next reaction without further characterization.

To a solution of DMP (4.65 g, 10.96 mmol) in CH₂Cl₂ (20 mL) was added a solution of alcohol, methyl 5-(5-hydroxypentyl)picolinate (1.3 g, 5.5 mmol) in CH₂Cl₂ (40 mL) at 0 °C, and the mixture was stirred for 4 hours at room temperature. The aldehyde product was extracted with organic solvent CH₂Cl₂, and the combined extracts were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. Silica gel was added, and the solvent evaporated to obtain a plug. Purification by column chromatography using 10%, 30% and 50% EtOAc in hexanes afforded aldehyde 22d (1.35 g) in 60% yield as a transparent oil. R_f = 0.51 (10% EtOAc/hexanes). The intermediate 22d was used for the next reaction without further characterization.

Ethyl 5-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinate (27d).

To a solution of aldehyde 22d (1.54 g, 6.2 mmol) in anhydrous Et₂O was added 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (932 mg, 3.09 mmol), 2 N HCl in Et₂O solution (0.3 mL, 0.62 mmol) and the mixture was stirred at room temperature for 24 hours.
TLC showed the disappearance of the starting material and formation of one major non-polar spot (hexane/EtOAc). The reaction solution was washed with 5% NaHCO$_3$ solution and extracted with H$_2$O and dried over Na$_2$SO$_4$. After evaporation of solvent, the residue 25d was used directly in the next step without further characterization. TLC R$_f$ = 0.9 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (384 mg, 3.04 mmol) and sodium acetate (454 mg, 5.53 mmol) in water (5 mL) and methanol (5 mL) was added α-bromo aldehyde 25d (830 mg, 2.8 mmol). The reaction mixture was stirred at 45 °C for 4 hours. TLC showed the disappearance of starting materials and the formation of one major pink spot (CHCl$_3$/MeOH). After evaporation of solvent, CH$_3$OH was added followed by silica gel and solvent was evaporated to afford a plug. The plug was loaded onto a silica gel column and eluted initially with CHCl$_3$ followed by gradual increase of 10% MeOH in CHCl$_3$. Fractions with the required R$_f$ were pooled and evaporated to afford 27d (740 mg, 20% over two steps) as a light pink sticky solid. TLC R$_f$ = 0.3 (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, DMSO-d$_6$) δ 10.67 (s, 1H, NH), 10.12 (s, 1H, NH), 8.55 (s, 1H, Ar), 7.97 (d, J = 8.1 Hz, 1H, Ar), 7.82 (d, J = 8.2 Hz, 1H, Ar), 6.39 (s, 1H, Ar), 6.00 (s, 2H, 2-NH$_2$), 4.48 (q, J = 7.2 Hz, 2H, CH$_2$), 2.69 (t, J = 7.7 Hz, 2H, CH$_2$), 2.58 (t, J = 7.4 Hz, 2H, CH$_2$), 1.94 (d, J = 7.5 Hz, 2H, CH$_2$), 1.36 (t, J = 7.1 Hz, 3H, CH$_3$). The intermediate 27d was used for the next reaction without further characterization.

5-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinic acid (28d).

To 27d (740 mg, 2.26 mmol), was added 1 N NaOH (2 mL) and the resulting mixture was stirred at rt for 1 h. TLC indicated the disappearance of starting material and the formation of
one major spot at the origin. The solution was cooled in an ice bath, and the pH was adjusted to 5 using 1 N HCl. The resulting suspension was chilled in a dry ice/acetone bath and thawed to 4 °C overnight in a refrigerator. The precipitate was filtered, washed with cold water, and dried in a desiccator under reduced pressure using P₂O₅ to yield 28d (670 mg, 94%) as a brown semisolid. TLC Rf = 0.3 (CHCl₃/MeOH, 10:1 in a drop of CH₃COOH); ¹H NMR (400 MHz, DMSO-d₆) δ 10.67 (s, 1H, NH), 10.13 (s, 1H, NH), 8.56 (d, J = 2.2 Hz, 1H, Ar), 7.97 (d, J = 8.0 Hz, 1H, Ar), 7.84 (dd, J = 8.1, 2.1 Hz, 1H, Ar), 6.37 (d, J = 2.1 Hz, 1H, Ar), 6.02 (s, 2H, 2NH₂), 2.70 (t, J = 7.6 Hz, 2H, CH₂), 2.59 (t, J = 7.5 Hz, 2H, CH₂), 1.96 (p, J = 7.6 Hz, 2H, CH₂). Anal. Calcd for C₁₅H₁₅N₅O₃ · 0.89 H₂O: C, 54.68; H, 5.13; N, 21.25. Found: C, 54.80; H, 5.27; N, 20.98.

Diethyl (5-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinoyl)-L-glutamate (29d).

A mixture of 28d (0.67 g, 2.14 mmol), N-methylmorpholine (0.28 mL, 2.57 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.45 g, 2.57 mmol) in anhydrous DMF in a round bottomed flask, was stirred for 1.5 h, at room temperature. Subsequently, N-methylmorpholine (0.28 mL, 2.57 mmol) and L-glutamic acid diethylester hydrochloride (0.65 g, 3.21 mmol) was added to the mixture and stirred at room temperature overnight until disappearance of the starting material and a new major non-polar spot was observed on TLC (hexane/EtOAc). After evaporation of the solvent in vacuo, MeOH was added followed by silica gel and the solvent was evaporated by further drying. The resulting plug was loaded on to a silica gel column and eluted with hexanes followed by gradual increase of EtOAc to 50% EtOAc in hexanes. Fractions with the desired Rf (TLC) were pooled and evaporated to afford 29d (0.7 g, 65%) as a yellow syrup. TLC Rf = 0.45 (CHCl₃/MeOH, 10:1); ¹H NMR (400 MHz, DMSO-d₆) δ 10.67 (s, 1H, NH), 10.14 (s, 1H, NH), 8.93 (d, J = 8.2 Hz, 1H, NH), 8.53 (d, J = 2.0 Hz, 1H, Ar), 7.94 (d, J = 8.0 Hz, 1H, Ar), 7.84 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 6.39 (d, J = 2.0 Hz, 1H, Ar), 6.01 (s,
2H, 2NH₂), 4.53 (ddd, J = 9.5, 8.0, 5.0 Hz, 1H, CH), 4.12 (qd, J = 7.1, 1.8 Hz, 2H, CH₂), 4.01 (q, J = 7.1 Hz, 2H, CH₂), 2.71 (t, J = 7.6 Hz, 2H, CH₂), 2.60 (t, J = 7.4 Hz, 2H, CH₂), 2.43 – 2.32 (m, 2H, CH₂), 2.23 – 2.03 (m, 2H, CH₂), 1.97 (q, J = 7.6 Hz, 2H, CH₂), 1.17 (t, 6H, CH₃).

The intermediate 29d was used for the next reaction without further characterization.

(5-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)picolinoyl)-1-glutamic acid (14c).

Compound 14c was prepared using the similar procedure to 28d, from 29d (0.7 g, 1.4 mmol) to give 14c (485 mg, 78%) as a brown powder. mp: 241.5 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.65 (s, 1H, NH), 10.11 (s, 1H, NH), 8.97 (d, J = 7.8 Hz, 1H, NH), 8.54 (d, J = 2.0 Hz, 1H, Ar), 7.91 (d, J = 8.1 Hz, 1H, Ar), 7.74 (dd, J = 8.3, 2.1 Hz, 1H, Ar), 6.42 (d, J = 2.2 Hz, 1H, Ar), 6.01 (s, 2H, 2-NH₂), 4.54 (m, 1H, CH), 2.78 (t, J = 7.6 Hz, 2H, CH₂), 2.57 (t, J = 7.1 Hz, 2H, CH₂), 2.45 – 2.37 (m, 2H, CH₂), 2.28 – 2.13 (m, 2H, CH₂), 2.05 (q, J = 7.6 Hz, 2H, CH₂). Anal. Calcd for C₂₀H₂₂N₆O₆· 1.7177 H₂O: C, 50.75; H, 5.42; N, 17.54. Found: C, 50.79; H, 5.37; N, 17.61.

Diethyl (5-bromopicolinoyl)-1-glutamate (30d).

To a solution of 5-bromopicolinic acid 20d (4 g, 19.8 mmol) in anhydrous DMF (20 mL) was added N-methylmorpholine (2.61 mL, 23.76 mmol) and 2-chloro-4,6- dimethoxy-1,3,5-triazine (4.17 g, 23.76 mmol). The resulting mixture was stirred at room temperature for 2 h. To this mixture was added N-methylmorpholine (2.61 mL, 23.76 mmol) and 1-glutamic acid diethyl ester hydrochloride (6.04 g, 29.7 mmol). The reaction mixture was stirred for an additional 4 h at room temperature. After evaporation of the solvent in vacuo, MeOH was added followed by silica gel and the solvent was evaporated by further drying. The resulting plug was loaded on to a silica gel column and eluted with hexanes followed by gradual increase of EtOAc to 30% EtOAc in hexanes. Fractions with the desired Rf (TLC) were pooled and evaporated to afford glutamate ester 30d (6.2 g, 80%) as a colorless syrup. TLC Rf = 0.56 (Hexane: EtOAc,
1H NMR (400 MHz, Chloroform-d) δ 8.67 (d, J = 4.4 Hz, 1H, Ar), 8.42 (d, J = 8.3 Hz, 1H, NH), 8.08 (d, J = 8.4 Hz, 1H, Ar), 8.04 – 7.97 (m, 1H, Ar), 4.82 (td, J = 8.1, 4.5 Hz, 1H, CH), 4.33 – 4.20 (m, 2H, CH2), 4.14 (p, J = 7.1 Hz, 2H, CH2), 2.52 – 2.33 (m, 2H, CH2), 2.20 (m, 2H, CH2), 1.33 (t, J = 8.7, 5.5 Hz, 3H, CH3), 1.26 (t, J = 7.1, 6.4 Hz, 3H, CH3). Mass calculated for C15H19BrN2O5 [M + Na]+, 409.04 Found 409.3. The intermediate 30d was used for the next reaction without further characterization.

Diethyl (6-bromonicotinoyl)-L-glutamate (47d).

Compound 47d was prepared using the similar procedure to 30d, from 6-bromonicotinic acid 46d (3 g, 14.85 mmol), N-methylmorpholine (1.96 mL, 17.82 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (3.13 g, 17.82 mmol) and L-glutamic acid diethyl ester hydrochloride (4.53 g, 22.28 mmol) to give 47d (4.79 g, 83%) as a clear oil. TLC Rf = 0.52 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 9.01 – 8.96 (d, J = 9.3 Hz, 1H, NH), 8.10 (dd, J = 8.1, 2.3 Hz, 1H, Ar), 7.47 – 7.44 (d, J = 2.1 Hz, 1H, Ar), 7.43 (d, J = 8.1 Hz, 1H, Ar), 4.77 (td, J = 7.7, 4.7 Hz, 1H, CH), 4.26 (qd, J = 7.2, 1.9 Hz, 2H, CH2), 4.14 (dtt, J = 9.3, 2.3 Hz, 2H, CH2), 3.73 (s, 2H, CH2), 2.37 – 2.12 (m, 2H, CH2), 1.32 (t, J = 7.1 Hz, 3H, CH3), 1.25 (t, J = 7.2 Hz, 3H, CH3). Mass calculated for C15H19BrN2O5 [M + Na]+, 409.04 Found 409.2. The intermediate 47d was used for the next reaction without further characterization.

Diethyl (5-(4-hydroxybut-1-yn-1-yl)picolinoyl)-L-glutamate (31d).

Compound 31d (2.35 g, 60%) was prepared using similar procedure to 23d, from diethyl (5-bromopicolinoyl)-L-glutamate, 30d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and but-3-yn-1-ol (0.94 mL, 12.4 mmol). TLC Rf = 0.48 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.68 (s, 1H, Ar), 8.55 (d, J = 8.5 Hz, 1H, NH), 8.12 (d, J = 8.0 Hz, 1H, Ar), 7.86 – 7.78 (m, 1H, Ar), 4.83 (dt, J = 8.2, 4.2 Hz, 1H, CH), 4.28 (q, J = 7.4, 2H, CH2), 4.12 (q, J = 7.0 Hz, 2H,
CH₂), 3.65 (dd, J = 6.4, 5.5 Hz, 2H, CH₂), 2.55 – 2.53 (m, 2H, CH₂), 2.52 – 2.32 (m, 4H, CH₂), 1.33 (t, J = 7.2 Hz, 3H, CH₃), 1.26 (t, J = 7.1 Hz, 3H, CH₃). The intermediate 31d was used for the next reaction without further characterization.

Diethyl (5-(6-hydroxyhex-1-yn-1-yl)picolinoyl)-L-glutamate (32d).

Compound 32d (2.82 g, 70%) was prepared using similar procedure to 23d, from diethyl (5-bromopicolinoyl)-L-glutamate, 30d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and hex-5-yn-1-ol (1.37 mL, 12.4 mmol). TLC Rf = 0.49 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 8.58 (s, 1H, Ar), 8.51 (d, J = 8.5 Hz, 1H, NH), 8.11 (d, J = 8.0 Hz, 1H, Ar), 7.86 – 7.81 (m, 1H, Ar), 4.84 (dt, J = 8.2, 4.2 Hz, 1H, CH), 4.26 (q, J = 7.2 Hz, 2H, CH₂), 4.12 (q, J = 7.0 Hz, 2H, CH₂), 3.75 (m, 2H, CH₂), 2.57 – 2.52 (m, 2H, CH₂), 2.52 – 2.32 (m, 4H, CH₂), 1.77 (t, J = 3.2 Hz, 4H, CH₂), 1.33 (t, J = 7.1 Hz, 3H, CH₃), 1.27 (t, J = 7.1 Hz, 3H, CH₃). The intermediate 32d was used for the next reaction without further characterization.

Diethyl (5-(7-hydroxyhept-1-yn-1-yl)picolinoyl)-L-glutamate (33d).

Compound 33d (2.9 g, 67%) was prepared using similar procedure to 23d, from diethyl (5-bromopicolinoyl)-L-glutamate, 30d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and hept-6-yn-1-ol (1.56 mL, 12.4 mmol). TLC Rf = 0.53 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 8.61 (s, 1H, Ar), 8.53 (d, J = 8.3 Hz, 1H, NH), 8.13 (d, J = 7.8 Hz, 1H, Ar), 7.85 (m, 1H, Ar), 4.81 (m, 1H, CH), 4.21 (q, J = 6.9 Hz, 2H, CH₂), 4.14 (q, J = 7.1 Hz, 2H, CH₂), 3.69 (dd, J = 6.4, 5.5 Hz, 2H, CH₂), 2.55 (m, 2H, CH₂), 2.51 – 2.35 (m, 4H, CH₂), 1.74 (m, 4H, CH₂), 1.52 (m, 2H, CH₂), 1.35 (t, J = 7.1 Hz, 3H, CH₃), 1.21 (t, J = 7.1 Hz, 3H, CH₃). The intermediate 33d was used for the next reaction without further characterization.
Diethyl (6-(5-hydroxypent-1-yn-1-yl)nicotinoyl)-L-glutamate (48d).

Compound 48d (2.23 g, 55%) was prepared using similar procedure to 23d, from diethyl (6-bromonicotinoyl)-L-glutamate, 47d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and hex-5-yn-1-ol (1.17 mL, 12.4 mmol). TLC $R_f$ = 0.44 (Hexane: EtOAc, 3:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.99 (d, $J = 8.6$ Hz, 1H, NH), 8.12 (dd, $J = 8.0$, 1.8 Hz, 1H, Ar), 7.49 – 7.43 (d, $J = 2.1$ Hz, 1H, Ar), 7.39 (d, $J = 8.3$ Hz, 1H, Ar), 4.75 (m, 1H, CH), 4.25 (q, $J = 7.2$ Hz, 2H, CH$_2$), 4.11 (q, $J = 9.3$ Hz, 2H, CH$_2$), 3.75 (t, 2H, CH$_2$), 2.57 – 2.43 (m, 4H, CH$_2$), 2.36 – 2.14 (m, 2H, CH$_2$), 1.81 – 1.74 (m, 2H, CH$_2$), 1.32 (t, $J = 7.1$ Hz, 3H, CH$_3$), 1.28 – 1.22 (m, 3H, CH$_3$). The intermediate 48d was used for the next reaction without further characterization.

Diethyl (6-(6-hydroxyhex-1-yn-1-yl)nicotinoyl)-L-glutamate (49d).

Compound 49d (2.65 g, 63%) was prepared using similar procedure to 23d, from diethyl (6-bromonicotinoyl)-L-glutamate, 47d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and hex-5-yn-1-ol (1.37 mL, 12.4 mmol). TLC $R_f$ = 0.47 (Hexane: EtOAc, 3:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.01 (d, $J = 9.1$ Hz, 1H, NH), 8.10 (dd, $J = 8.2$, 2.1 Hz, 1H, Ar), 7.45 (d, $J = 2.4$ Hz, 1H, Ar), 7.41 (d, $J = 7.9$ Hz, 1H, Ar), 4.73 (m, $J = 7.9$ Hz, 1H, CH), 4.23 (q, $J = 7.7$ Hz, 2H, CH$_2$), 4.13 (q, $J = 8.8$ Hz, 2H, CH$_2$), 3.69 (t, 2H, CH$_2$), 2.59 – 2.47 (m, 4H, CH$_2$), 2.28 (m, 2H, CH$_2$), 1.84 – 1.77 (m, 2H, CH$_2$), 1.56 – 1.47 (m, 2H, CH$_2$), 1.33 (t, $J = 7.1$ Hz, 3H, CH$_3$), 1.25 (t, 3H, CH$_3$). Mass calculated for C$_{21}$H$_{28}$N$_2$O$_6$ [M + H]$^+$, 405.2 Found 405.4. The intermediate 49d was used for the next reaction without further characterization.

Diethyl (6-(7-hydroxyhept-1-yn-1-yl)nicotinoyl)-L-glutamate (50d).
Compound 50d (2.53 g, 58%) was prepared using similar procedure to 23d, from diethyl (6-bromonicotinoyl)-L-glutamate, 47d (4 g, 10.33 mmol) in anhydrous acetonitrile was added palladium chloride (73.27 mg, 0.41 mmol), triphenylphosphine (108 mg, 0.41 mmol), copper iodide (315 mg, 1.65 mmol), triethylamine (14.4 mL, 103.3 mmol), and hept-6-yn-1-ol (1.56 mL, 12.4 mmol). TLC R_f = 0.51 (Hexane: EtOAc, 3:1); ^1H NMR (400 MHz, Chloroform-d) δ 9.00 – 8.95 (d, J = 9.0 Hz, 1H, NH), 8.11 (dd, J = 8.1, 2.4 Hz, 1H, Ar), 7.46 – 7.43 (d, J = 2.1 Hz, 1H, Ar), 7.40 (d, J = 8.2 Hz, 1H, Ar), 4.75 (td, J = 8.1, 4.4 Hz, 1H, CH), 4.26 (q, J = 7.9, Hz, 1H, CH2), 4.14 (q, J = 8.8 Hz, 2H, CH2), 3.74 (t, 2H, CH2), 2.57 – 2.43 (m, 4H, CH2), 2.29 (m, 2H, CH2), 1.81 – 1.74 (m, 2H, CH2), 1.59 – 1.45 (m, 4H, CH2), 1.32 (t, J = 7.1 Hz, 3H, CH3), 1.28 – 1.22 (m, 3H, CH3). The intermediate 50d was used for the next reaction without further characterization.

Methyl 5-(3-hydroxyprop-1-yn-1-yl)picolinate (71d).

Compound 71d (1.18 g, 66%) was prepared using similar procedure to 23d, from methyl 5-bromopicolinate, 70d (2 g, 9.26 mmol) in anhydrous acetonitrile was added palladium chloride (65.66 mg, 0.37 mmol), triphenylphosphine (97.13 mg, 0.37 mmol), copper iodide (282 mg, 1.48 mmol), triethylamine (12.9 mL, 92.58 mmol), and but-3-yn-1-ol (0.64 mL, 11.11 mmol). TLC R_f = 0.51 (Hexane: EtOAc, 3:1) ^1H NMR (400 MHz, Chloroform-d) δ 8.73 (dd, J = 2.2, 0.8 Hz, 1H, Ar), 8.09 (dd, J = 8.1, 0.9 Hz, 1H, Ar), 7.83 (dd, J = 8.1, 2.1 Hz, 1H, Ar), 4.03 (s, 3H, OCH3), 3.97 (t, J = 7.1 Hz, 1H, OH), 3.77(t, J = 5.9 Hz, 2H, CH2). The intermediate 71d was used for the next reaction without further characterization.

Methyl 5-(4-hydroxybut-1-yn-1-yl)picolinate (72d).

Compound 72d (1.34 g, 70%) was prepared using similar procedure to 23d, from methyl 5-bromopicolinate, 70d (2 g, 9.26 mmol) in anhydrous acetonitrile was added palladium chloride (65.66 mg, 0.37 mmol), triphenylphosphine (97.13 mg, 0.37 mmol), copper iodide (282 mg, 1.48 mmol), triethylamine (12.9 mL, 92.58 mmol), and propargyl alcohol (0.84 mL, 11.11
mmol). TLC R_f = 0.49 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.79 (dd, J = 2.1, 0.9 Hz, 1H, Ar), 8.13 (dd, J = 8.3, 0.8 Hz, 1H, Ar), 7.89 (dd, J = 8.2, 2.4 Hz, 1H, Ar), 4.11 (q, J = 7.0 Hz, 1H, OH), 4.01 (s, 3H, OCH_3), 3.85 (q, J = 6.9 Hz, 2H, CH_2), 2.68 (t, J = 7.0 Hz, 2H, CH_2). The intermediate 72d was used for the next reaction without further characterization.

Methyl 5-(5-hydroxypent-1-yn-1-yl)picolinate (73d).

Compound 73d (1.28 g, 63%) was prepared using similar procedure to 23d, from methyl 5-bromopicolinate, 70d (2 g, 9.26 mmol) in anhydrous acetonitrile was added palladium chloride (65.66 mg, 0.37 mmol), triphenylphosphine (97.13 mg, 0.37 mmol), copper iodide (282 mg, 1.48 mmol), triethylamine (12.9 mL, 92.58 mmol), and pent-4-yn-1-ol (1.03 mL, 11.11 mmol). TLC R_f = 0.54 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.76 (dd, J = 2.4, 0.7 Hz, 1H, Ar), 8.13 (dd, J = 8.2, 0.8 Hz, 1H, Ar), 7.81 (dd, J = 8.2, 1.8 Hz, 1H, Ar), 4.14 (q, J = 6.8 Hz, 1H, OH), 4.03 (s, 3H, OCH_3), 3.87 (q, J = 7.0 Hz, 2H, CH_2), 2.63 (t, J = 7.0 Hz, 2H, CH_2), 1.92 (tt, J = 7.0, 6.1 Hz, 2H, CH_2). Mass calculated for C_{12}H_{13}NO_3 [M + H]^+, 220.09 Found 220.2. The intermediate 73d was used for the next reaction without further characterization.

Diethyl (5-(4-hydroxybutyl)picolinoyl)-L-glutamate (34d).

Compound 34d (3.35 g, 87%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 31d (3.8 g, 10.10 mmol), 10% palladium on activated carbon (4 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC R_f = 0.46 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.58 (d, J = 1.9 Hz, 1H, Ar), 8.53 (d, J = 8.5 Hz, 1H, NH), 8.12 (d, J = 7.8 Hz, 1H, Ar), 7.68 (dd, J = 7.9, 1.9 Hz, 1H, Ar), 4.84 (td, J = 8.3, 4.7 Hz, 1H, CH), 4.25 (qd, J = 7.1, 1.6 Hz, 2H, CH_2), 4.11 (q, J = 7.2 Hz, 2H, CH_2), 3.69 (t, J = 6.2 Hz, 2H, CH_2), 2.74 (t, J = 7.6 Hz, 2H, CH_2), 2.59 – 2.29 (m, 4H, CH_2), 1.75 (q, J = 7.4 Hz,
Diethyl (5-(6-hydroxyhexyl)picolinoyl)-L-glutamate (35d).

Compound 35d (3.8 g, 90%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 32d (4.2 g, 10.38 mmol), 10% palladium on activated carbon (4 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.47 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.52 (d, J = 8.5 Hz, 1H, NH), 8.43 (d, J = 1.8 Hz, 1H, Ar), 8.10 (d, J = 7.7 Hz, 1H, Ar), 7.67 (dd, J = 7.8, 2.1 Hz, 1H, ar), 4.83 (m, 1H, CH), 4.28 (q, J = 7.2, 2H, CH₂), 4.11 (q, J = 7.4 Hz, 2H, CH₂), 3.64 (t, J = 5.9 Hz, 2H, CH₂), 2.69 (t, J = 7.2 Hz, 2H, CH₂), 2.42 (m, 4H, CH₂), 1.73 (m, 4H, CH₂), 1.69 – 1.61 (m, 4H, CH₂), 1.33 (t, J = 7.1 Hz, 3H, CH₃). The intermediate 35d was used for the next reaction without further characterization.

Diethyl (5-(7-hydroxyheptyl)picolinoyl)-L-glutamate (36d).

Compound 36d (4.23 g, 93%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 33d (4.5 g, 10.75 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.50 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.57 (d, J = 1.9 Hz, 1H, Ar), 8.55 (d, J = 8.5 Hz, 1H, NH), 8.16 (d, J = 7.8 Hz, 1H, Ar), 7.65 (dd, J = 7.9, 1.9 Hz, 1H, Ar), 4.85 (t, J = 8.3, 4.7 Hz, 1H, CH), 4.25 (qd, J = 7.1, 1.6 Hz, 2H, CH₂), 4.11 (q, J = 7.2 Hz, 2H, CH₂), 3.69 (t, J = 6.2 Hz, 2H, CH₂), 2.74 (t, J = 7.6 Hz, 2H, CH₂), 2.59 – 2.29 (m, 4H, CH₂), 1.76-1.72 (m, 4H, CH₂), 1.69 – 1.61 (m, 4H, CH₂), 1.57 (m, 2H, CH₂), 1.33 (t, J = 7.1 Hz, 3H, CH₃), 1.25 (t, J = 7.1 Hz, 3H, CH₃). Mass calculated for C₂₂H₃₀N₂O₆ [M + H]^+, 445.23 Found 445.4. The intermediate 36d was used for the next reaction without further characterization.

Diethyl (6-(5-hydroxypentyl)nicotinoyl)-L-glutamate (51d).
Compound 51d (3.83 g, 88%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 48d (4.3 g, 11.01 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.41 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.97 (d, J = 9.1 Hz, 1H, NH), 8.12 (dd, J = 8.0, 2.0 Hz, 1H, Ar), 7.53 – 7.48 (d, J = 2.1 Hz, 1H, Ar), 7.44 (d, J = 8.3 Hz, 1H, Ar), 4.75 (td, J = 7.9, 4.6 Hz, 1H, CH), 4.25 (q, J = 7.2 Hz, 2H, CH2), 4.12 (q, J = 9.3 Hz, 2H, CH2), 3.76 (t, 2H, CH2), 2.72 (m, 2H, CH2) 2.59 – 2.46 (m, 4H, CH2), 2.39 – 2.18 (m, 2H, CH2), 1.81 – 1.74 (m, 2H, CH2), 1.68 – 1.55 (m, 2H, CH2), 1.34 (t, J = 7.1 Hz, 3H, CH3), 1.25 – 1.20 (m, 3H, CH3). The intermediate 51d was used for the next reaction without further characterization.

Diethyl (6-(6-hydroxyhexyl)nicotinoyl)-L-glutamate (52d).

Compound 52d (395 g, 95%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 49d (4.1 g, 10.14 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.46 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.91 (d, J = 8.9 Hz, 1H, NH), 8.07 (dd, J = 8.4, 2.1 Hz, 1H, Ar), 7.54 – 7.48 (d, J = 2.2 Hz, 1H, Ar), 7.37 (d, J = 7.8 Hz, 1H, Ar), 4.59 (m, 1H, CH), 4.27 (q, J = 7.6 Hz, 2H, CH2), 4.14 (q, J = 9.0 Hz, 2H, CH2), 3.71 (t, 2H, CH2), 2.69 (m, 2H, CH2), 2.55 – 2.44 (m, 4H, CH2), 2.39 – 2.20 (m, 2H, CH2), 1.77 – 1.73 (m, 2H, CH2), 1.69 – 1.51 (m, 4H, CH2), 1.33 (t, J = 7.1 Hz, 3H, CH3), 1.28 (m, 3H, CH3). Mass calculated for C21H32N2O6 [M + H]+, 409.23 Found 409.4. The intermediate 52d was used for the next reaction without further characterization.

Diethyl (6-(7-hydroxyheptyl)nicotinoyl)-L-glutamate (53d).

Compound 53d (3.42 g, 87%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 50d (3.9 g, 9.32 mmol), 10% palladium on activated carbon (2 g) in
a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.48 (Hexane: EtOAc, 3:1)

1H NMR (400 MHz, Chloroform-d) δ 8.98 (d, J = 9.1 Hz, 1H, NH), 8.14 (dd, J = 7.9, 2.2 Hz, 1H, Ar), 7.75 - 7.51 (d, J = 2.1 Hz, 1H, Ar), 7.45 (d, J = 8.4 Hz, 1H, Ar), 4.68 (m, 1H, CH), 4.29 (q, J = 7.3 Hz, 2H, CH2), 4.12 (q, J = 8.8 Hz, 2H, CH2), 3.64 (t, 2H, CH2), 2.71 (m, 2H, CH2) 2.53 – 2.44 (m, 4H, CH2), 2.40 – 2.21 (m, 2H, CH2), 1.78 – 1.71 (m, 2H, CH2), 1.64 – 1.52 (m, 4H, CH2), 1.49 (m, 2H, CH2), 1.31 (t, J = 7.1 Hz, 3H, CH3), 1.26 – 1.19 (m, 3H, CH3).
The intermediate 53d was used for the next reaction without further characterization.

Methyl 5-(3-hydroxypropyl)picolinate (74d).

Compound 74d (2.25 g, 88%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 71d (2.5 g, 13.08 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.51 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.62 (d, J = 2.2 Hz, 1H, Ar), 8.08 (dd, J = 8.1, 0.7 Hz, 1H, Ar), 7.69 (dd, J = 8.0, 1.1 Hz, 1H, Ar), 4.09 (s, 3H, OCH3), 4.49 (q, J = 7.1 Hz, 2H, CH2), 2.88 – 2.77 (m, 2H, CH2), 1.98 – 1.86 (m, 2H, CH2). Mass calculated for C10H14NO3 [M + H]+, 196.10 Found 196.2. The intermediate 74d was used for the next reaction without further characterization.

Methyl 5-(4-hydroxybutyl)picolinate (75d).

Compound 75d (2.49 g, 81%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 72d (3 g, 14.62 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.47 (Hexane: EtOAc, 3:1) 1H NMR (400 MHz, Chloroform-d) δ 8.58 (m, 1H, Ar), 8.07 – 8.01 (m, 1H, Ar), 7.72 – 7.60 (m, 1H, Ar), 4.02 (s, 3H, OCH3), 3.69 – 3.63 (m, 2H, CH2), 2.76 – 2.68 (m, 2H, CH2), 1.79 – 1.70 (m, 2H, CH2), 1.64 (m, 2H, CH2). Mass calculated for C10H14NO3 [M + H]+, 196.10 Found 196.2. The intermediate 75d was used for the next reaction without further characterization.

Methyl 5-(5-hydroxypentyl)picolinate (76d).
Compound 76d (2.51 g, 75%) was prepared using similar catalytic hydrogenation procedure to 22d (condition B) from 73d (3.3 g, 15.05 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC R_f = 0.54 (Hexane: EtOAc, 3:1) \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 8.60 – 8.55 (m, 1H, Ar), 8.10 – 8.04 (m, 1H, Ar), 7.69 – 7.62 (m, 1H, Ar), 4.01 (s, 3H, OCH\(_3\)), 3.68 – 3.61 (m, 2H, CH\(_2\)), 2.76 – 2.67 (m, 2H, CH\(_2\)), 1.76 – 1.66 (m, 2H, CH\(_2\)), 1.62 (m, 2H, CH\(_2\)), 1.49 – 1.40 (m, 2H, CH\(_2\)). The intermediate 76d was used for the next reaction without further characterization.

Diethyl (5-(4-oxobutyl)picolinoyl)-L-glutamate (37d).

Compound 37d (1.62 g, 65%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 34d (2.5 g, 6.57 mmol) of DMP (5.57 g, 13.14 mmol) in CH\(_2\)Cl\(_2\) (20 mL). TLC R_f = 0.66 (Hexane: EtOAc, 3:1) \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 9.76 (t, J = 7.2 Hz, 1H, CHO), 8.46 (d, J = 8.5 Hz, 1H, NH), 8.38 (t, J = 2.2 Hz, 1H, Ar), 8.06 (dd, J = 7.9, 4.1 Hz, 1H, Ar), 7.64 (dt, J = 8.0, 2.3 Hz, 1H, Ar), 4.78 (tt, J = 8.4, 4.1 Hz, 1H, CH), 4.24 – 4.16 (m, 2H, CH\(_2\)), 4.10 (q, J = 7.2 Hz, 2H, CH\(_2\)), 2.70 (t, J = 7.8 Hz, 2H, CH\(_2\)), 2.49 (td, J = 7.0, 4.4 Hz, 2H, CH\(_2\)), 2.44 – 2.26 (m, 4H, CH\(_2\)), 1.98 – 1.90 (m, 2H, CH\(_2\)), 1.32 – 1.12 (m, 6H, CH\(_3\)). The intermediate 37d was used for the next reaction without further characterization.

Diethyl (5-(6-oxohexyl)picolinoyl)-L-glutamate (38d).

Compound 38d (1.38 g, 68%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 35d (2.2 g, 5.39 mmol) of DMP (4.57 g, 10.77 mmol) in CH\(_2\)Cl\(_2\) (20 mL). TLC R_f = 0.69 (Hexane: EtOAc, 3:1) \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 9.85 (t, J = 7.1 Hz, 1H, CHO), 8.56 (d, J = 8.3 Hz, 1H, NH), 8.39 (t, J = 2.3 Hz, 1H, Ar), 8.12 (dd, J = 7.8, 4.2 Hz, 1H, Ar), 7.77 (dt, J = 7.9, 2.0 Hz, 1H, Ar), 4.81 (tt, J = 8.3, 4.0 Hz, 1H, CH), 4.20 – 4.15 (m, 2H, CH\(_2\)), 4.10 (q, J = 7.4 Hz, 2H, CH\(_2\)), 2.72 (t, J = 7.3 Hz, 2H, CH\(_2\)), 2.52 (td, J = 6.7, 4.2 Hz, 2H, CH\(_2\)), 2.47 – 2.23 (m, 4H, CH\(_2\)), 2.15 – 2.01 (m, 2H, CH\(_2\)), 1.95 – 1.87 (m, 4H, CH\(_2\)),...
The intermediate 38d was used for the next reaction without further characterization.

Diethyl (5-(7-oxoheptyl)picolinoyl)-L-glutamate (39d).

Compound 39d (1.19 g, 66%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 36d (1.8 g, 4.26 mmol) of DMP (3.61 g, 8.52 mmol) in CH₂Cl₂ (20 mL).

TLC Rf = 0.67 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 9.81 (t, J = 6.8 Hz, 1H, CHO), 8.48 (d, J = 8.1 Hz, 1H, NH), 8.36 (t, J = 2.4 Hz, 1H, Ar), 7.89 (dd, J = 8.0, 3.8 Hz, 1H, Ar), 7.62 (dt, J = 7.7, 2.1 Hz, 1H, Ar), 4.85 (tt, J = 8.0, 3.8 Hz, 1H, CH), 4.21 – 4.12 (m, 2H, CH₂), 4.07 (q, J = 7.2 Hz, 2H, CH₂), 2.70 (t, J = 7.8 Hz, 2H, CH₂), 2.48 (td, J = 7.4, 4.4 Hz, 2H, CH₂), 2.47 – 2.23 (m, 4H, CH₂), 2.18 – 2.05 (m, 2H, CH₂), 1.98 – 1.82 (m, 4H, CH₂), 1.68 (m, 2H, CH₂), 1.33 – 1.10 (m, 6H, CH₃). Mass calculated for C₂₂H₃₂N₂O₆ [M + K]+, 459.2 Found 459.4. The intermediate 39d was used for the next reaction without further characterization.

Diethyl (6-(5-oxopentyl)nicotinoyl)-L-glutamate (54d).

Compound 54d (0.99 g, 47%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 51d (2.1 g, 5.32 mmol) of DMP (4.52 g, 10.65 mmol) in CH₂Cl₂ (20 mL).

TLC Rf = 0.65 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 9.76 (t, J = 7.2 Hz, 1H, CHO), 9.01 – 8.96 (d, J = 9.1 Hz, 1H, NH), 8.12 (dd, J = 7.8, 2.1 Hz, 1H, Ar), 7.51 (d, J = 2.4 Hz, 1H, Ar), 7.41 (d, J = 8.2 Hz, 1H, Ar), 4.68 (m, 1H, CH), 4.26 (q, J = 7.2 Hz, 2H, CH₂), 4.13 (q, J = 8.7 Hz, 2H, CH₂), 2.86 (t, 2H, CH₂), 2.55-2.41 (m, 4H, CH₂), 2.35 – 2.19 (m, 2H, CH₂), 1.78 – 1.71 (m, 2H, CH₂), 1.67 – 1.59 (m, 2H, CH₂), 1.35 (t, J = 7.1 Hz, 3H, CH₃), 1.26 – 1.21 (m, 3H, CH₃). The intermediate 54d was used for the next reaction without further characterization.

Diethyl (6-(6-oxohexyl)nicotinoyl)-L-glutamate (55d).
Compound 55d (1.18 g, 53%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 52d (2.25 g, 5.51 mmol) of DMP (4.67 g, 11.02 mmol) in CH$_2$Cl$_2$ (20 mL). TLC $R_f = 0.64$ (Hexane: EtOAc, 3:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.77 (t, J = 7.1 Hz, 1H, CHO), 8.98 (s, 1H, NH), 8.07 (dd, J = 8.1, 2.1 Hz, 1H, Ar), 7.32 (d, J = 7.1 Hz, 1H, Ar), 7.25 (d, J = 9.1 Hz, 1H, Ar), 4.78 (td, J = 7.9, 4.5 Hz, 1H, CH), 4.31 – 4.20 (m, 2H, CH$_2$), 4.18 – 4.07 (m, 2H, CH$_2$), 2.86 (t, J = 7.7 Hz, 2H, CH$_2$), 2.59 – 2.40 (m, 4H, CH$_2$), 2.37 – 2.11 (m, 2H, CH$_2$), 1.79 (m, 2H, CH$_2$), 1.73 – 1.64 (m, 2H, CH$_2$), 1.46 – 1.36 (m, 2H, CH$_2$), 1.31 (t, J = 7.1 Hz, 3H, CH$_3$), 1.27 – 1.20 (m, 3H, CH$_3$). The intermediate 55d was used for the next reaction without further characterization.

Diethyl (6-(7-oxoheptyl)nicotinoyl)-l-glutamate (56d).

Compound 56d (1.44 g, 60%) was prepared using similar DMP oxidation procedure to 22d (condition B) from 53d (2.4 g, 5.68 mmol) of DMP (4.82 g, 11.36 mmol) in CH$_2$Cl$_2$ (20 mL). TLC $R_f = 0.68$ (Hexane: EtOAc, 3:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 9.81 (t, J = 7.1 Hz, 1H, CHO), 8.93 (s, 1H, NH), 8.09 (dd, J = 8.1, 2.3 Hz, 1H, Ar), 7.36 (d, J = 7.2 Hz, 1H, Ar), 7.28 (d, J = 8.3 Hz, 1H, Ar), 4.74 (m, 1H, CH), 4.28 (m, 2H, CH$_2$), 4.20 – 4.11 (m, 2H, CH$_2$), 2.83 (t, J = 7.5 Hz, 2H, CH$_2$), 2.55 – 2.41 (m, 4H, CH$_2$), 2.39 – 2.14 (m, 2H, CH$_2$), 1.83 (m, 2H, CH$_2$), 1.75 – 1.68 (m, 2H, CH$_2$), 1.49 – 1.38 (m, 4H, CH$_2$), 1.33 (t, J = 7.1 Hz, 3H, CH$_3$), 1.26 – 1.22 (t, J = 7.2 Hz, 3H, CH$_3$). The intermediate 56d was used for the next reaction without further characterization.

Diethyl (6-(7-oxoheptyl)nicotinoyl)-l-glutamate (56d).

Compound 43d was prepared using the similar procedure to 27d from the aldehyde 37d (1.62 g, 4.28 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (646 mg, 2.14 mmol), and 2 N HCl in Et$_2$O solution (0.21 mL, 0.43 mmol). After the workup, the residue
40d was used directly in the next step without further characterization. TLC Rf = 0.88 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (950 mg, 7.54 mmol) and sodium acetate (1.12 g, 13.7 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 40d (1.96 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 43d (520 mg, 25% over two steps) as a light pink sticky solid. TLC Rf = 0.31 (CHCl3/MeOH, 10:1); 1H NMR (400 MHz, DMSO-d6) δ 10.58 (s, 1H, NH), 10.15 (s, 1H, NH), 8.92 (d, J = 7.9 Hz, 1H, NH), 8.52 (d, J = 2.2 Hz, 1H, Ar), 7.93 (d, J = 8.0 Hz, 1H, Ar), 7.83 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 6.35 (d, J = 1.9 Hz, 1H, Ar), 5.97 (s, 2H, 2-NH2), 4.55 (m, J = 8.9, 5.0 Hz, 1H, CH), 4.12 (qd, J = 7.0, 1.8 Hz, 2H, CH2), 4.01 (q, J = 7.1 Hz, 2H, CH2), 2.71 (m, 2H, CH2), 2.60 (s, 2H, CH2), 2.40 - 2.33 (m, 2H, CH2), 2.21 − 2.00 (m, 2H, CH2), 1.22 (t, J = 7.1 Hz, 3H, CH3), 1.11 (t, J = 7.1 Hz, 3H, CH3). The intermediate 43d was used for the next reaction without further characterization.

Diethyl (5-(4-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamate (44d).

Compound 44d was prepared using the similar procedure to 27d from the aldehyde 38d (1.38 g, 3.4 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (512.5 mg, 1.7 mmol), and 2 N HCl in Et2O solution (0.17 mL, 0.34 mmol). After the workup, the residue 41d was used directly in the next step without further characterization. TLC Rf = 0.90 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (728 mg, 5.78 mmol) and sodium acetate (862 mg, 10.50 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 41d (1.65 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 44d (698 mg, 40% over two steps) as a light pink sticky solid. TLC Rf = 0.33 (CHCl3/MeOH, 10:1); 1H NMR (400 MHz, DMSO-d6) δ 10.63 (s, 1H, NH), 10.12 (s, 1H, NH), 8.68 (d, J = 8.2 Hz, 1H, NH), 8.29 (d, J = 8.2 Hz, 1H, NH), 7.93 (d, J = 8.2 Hz, 1H, NH), 7.83 (dd, J = 8.2, 2.2 Hz, 1H, NH), 6.35 (d, J = 1.9 Hz, 1H, NH), 5.97 (s, 2H, 2-NH2), 4.55 (m, J = 8.9, 5.0 Hz, 1H, CH), 4.12 (qd, J = 7.0, 1.8 Hz, 2H, CH2), 4.01 (q, J = 7.1 Hz, 2H, CH2), 2.71 (m, 2H, CH2), 2.60 (s, 2H, CH2), 2.40 − 2.33 (m, 2H, CH2), 2.21 − 2.00 (m, 2H, CH2), 1.22 (t, J = 7.1 Hz, 3H, CH3), 1.11 (t, J = 7.1 Hz, 3H, CH3). The intermediate 43d was used for the next reaction without further characterization.

Diethyl (5-(4-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamate (44d).
Hz, 1H, NH), 8.55 (d, J = 2.4 Hz, 1H, Ar), 7.88 (d, J = 7.9 Hz, 1H, Ar), 7.81 (dd, J = 8.3, 2.2 Hz, 1H, Ar), 6.27 (d, J = 2.3 Hz, 1H, Ar), 5.93 (s, 2H, 2-NH₂), 4.58 (m, J = 8.6, 4.9 Hz, 1H, CH), 4.16 (q, J = 7.0 Hz, 2H, CH₂), 4.05 (q, J = 7.3 Hz, 2H, CH₂), 2.67 (m, 2H, CH₂), 2.58 (m, 2H, CH₂), 2.42 – 2.34 (m, 2H, CH₂), 2.26 – 2.11 (m, 2H, CH₂), 1.68 – 1.56 (m, 4H, CH₂), 1.18 (t, J = 6.8 Hz, 3H, CH₃), 1.13 (t, J = 7.3 Hz, 3H, CH₃). The intermediate 22d was used for the next reaction without further characterization.

Diethyl (5-(5-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)picolinoyl)-L-glutamate (45d).

Compound 45d was prepared using the similar procedure to 27d from the aldehyde 39d (1.19 g, 2.83 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (427 mg, 1.41 mmol), and 2 N HCl in Et₂O solution (0.14 mL, 0.28 mmol). After the workup, the residue 42d was used directly in the next step without further characterization. TLC Rf = 0.89 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (596 mg, 4.73 mmol) and sodium acetate (705 mg, 8.59 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 42d (1.41 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 45d (489 mg, 33% over two steps) as a light pink sticky solid. TLC Rf = 0.33 (CHCl₃/MeOH, 10:1); ¹H NMR (400 MHz, DMSO-d₆) δ 10.61 (s, 1H, NH), 10.11 (s, 1H, NH), 8.77 (d, J = 8.0 Hz, 1H, NH), 8.53 (d, J = 2.1 Hz, 1H, Ar), 7.92 (d, J = 8.4 Hz, 1H, Ar), 7.86 – 7.81 (m, 1H, Ar), 6.33 (d, J = 1.7 Hz, 1H, Ar), 5.96 (s, 2H, 2-NH₂), 4.52 (m, J = 8.1, 4.7 Hz, 1H, CH), 4.12 (q, J = 7.2, 2H, CH₂), 4.01 (q, J = 7.3 Hz, 2H, CH₂), 2.69 (m, 2H, CH₂), 2.56 (m, 2H, CH₂), 2.38 (m, 2H, CH₂), 2.13 (m, 2H, CH₂), 1.62 (m, 4H, CH₂), 1.19 (t, J = 7.0 Hz, 3H, CH₃), 1.14 (t, J = 7.3 Hz, 3H, CH₃). The intermediate 45d was used for the next reaction without further characterization.
Diethyl (6-(3-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)nicotinoyl)-l-glutamate (60d).

Compound 60d was prepared using the similar procedure to 27d from the aldehyde 54d (0.99 g, 2.90 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (368 mg, 1.22 mmol), and 2 N HCl in Et2O solution (0.12 mL, 0.24 mmol). After the workup, the residue 57d was used directly in the next step without further characterization. TLC Rf = 0.90 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (545 mg, 4.32 mmol) and sodium acetate (645 mg, 7.86 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 57d (1.18 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 60d (252 mg, 20% over two steps) as a light brown sticky solid. TLC Rf = 0.34 (CHCl3/MeOH, 10:1); 1H NMR (400 MHz, DMSO-d6) δ 10.48 (s, 1H, NH), 10.07 (s, 1H, NH), 8.86 (d, J = 8.3 Hz, 1H, NH), 8.12 (dd, J = 7.7, 2.3 Hz, 1H, Ar), 7.32 (d, J = 7.2 Hz, 1H, Ar), 7.25 (d, J = 7.8 Hz, 1H, Ar), 6.37 (d, J = 2.3 Hz, 1H, Ar), 5.56 (s, 2H, 2-NH2), 4.71 (td, J = 7.9, 5.0 Hz, 1H, CH), 4.31 – 4.20 (m, 2H, CH2), 4.18 – 4.07 (m, 2H, CH2), 2.72 (m, 2H, CH2), 2.58 (m, 2H, CH2), 2.37 (m, 2H, CH2), 1.64 – 1.52 (m, 4H, CH2), 1.31 (t, J = 7.1 Hz, 3H, CH3), 1.26 (m, 3H, CH3). The intermediate 60d was used for the next reaction without further characterization.

Diethyl (6-(4-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)nicotinoyl)-l-glutamate (61d).

Compound 61d was prepared using the similar procedure to 27d from the aldehyde 55d (1.18 g, 2.90 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (438 mg, 1.45 mmol), and 2 N HCl in Et2O solution (0.15 mL, 0.29 mmol). After the workup, the residue 58d was used directly in the next step without further characterization. TLC Rf = 0.89 (hexane/EtOAc 1:1)
To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (623 mg, 4.94 mmol) and sodium acetate (736 mg, 8.98 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 58d (1.41 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 61d (420 mg, 28% over two steps) as a light brown sticky solid. TLC Rf = 0.31 (CHCl3/MeOH, 10:1); 1H NMR (400 MHz, DMSO-d6) δ 10.57 (s, 1H, NH), 10.18 (s, 1H, NH), 8.74 (d, J = 8.2 Hz, 1H, NH), 8.09 (dd, J = 8.1, 2.3 Hz, 1H, Ar), 7.34 (d, J = 7.4 Hz, 1H, Ar), 7.27 (d, J = 7.8 Hz, 1H, Ar), 6.14 (d, J = 2.1 Hz, 1H, Ar), 5.81 (s, 2H, 2-NH2), 4.56 (td, J = 7.6, 4.7 Hz, 1H, CH), 4.27 – 4.18 (m, 2H, CH2), 4.18 – 4.09 (m, 2H, CH2), 2.83 (m, 2H, CH2), 2.57 (m, 2H, CH2), 2.42 – 2.36 (m, 2H, CH2), 1.72 – 1.51 (m, 4H, CH2), 1.42 – 1.34 (m, 2H, CH2), 1.32 (t, J = 6.8 Hz, 3H, CH3), 1.27 – 1.20 (m, 3H, CH3). The intermediate 61d was used for the next reaction without further characterization.

Diethyl (6-(5-(2-amino-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)nicotinoyl)-L-glutamate (62d).

Compound 62d was prepared using the similar procedure to 27d from the aldehyde 56d (1.44 g, 3.42 mmol) and 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane 24d (517 mg, 1.71 mmol), and 2 N HCl in Et2O solution (0.17 mL, 0.28 mmol). After the workup, the residue 59d was used directly in the next step without further characterization. TLC Rf = 0.91 (hexane/EtOAc 1:1)

To a solution of 2,6-diaminopyrimidin-4(3H)-one 26d (723 mg, 5.73 mmol) and sodium acetate (855 mg, 10.42 mmol) in water (5 mL) and methanol (5 mL) was added crude α-bromo aldehyde 59d (1.71 g). The reaction mixture was stirred at 45 °C for 4 hours to afford 62d (470 mg, 26% over two steps) as a light brown sticky solid. TLC Rf = 0.35 (CHCl3/MeOH, 10:1); 1H NMR (400 MHz, DMSO-d6) δ 10.61 (s, 1H, NH), 10.17 (s, 1H, NH), 8.87 (d, J = 8.2 Hz, 1H, NH), 8.14 (dd, J = 8.1, 2.2 Hz, 1H, Ar), 7.38 (d, J = 7.1 Hz, 1H, Ar), 7.24 (d, J = 8.1 Hz, 1H, Ar), 6.38 (d, J = 2.1 Hz, 1H, Ar), 6.01 (s, 2H, 2-NH2), 4.72 (td, J = 7.9, 4.9 Hz, 1H,
CH), 4.29 (m, 2H, CH₂), 4.15 – 4.10 (m, 2H, CH₂), 2.84 (m, 2H, CH₂), 2.57 (m, 2H, CH₂), 2.38 (m, 2H, CH₂), 1.71 – 1.63 (m, 4H, CH₂), 1.42 – 1.36 (m, 4H, CH₂), 1.31 (t, J = 7.1 Hz, 3H, CH₃), 1.24 – 1.18 (m, 3H, CH₃). The intermediate 62d was used for the next reaction without further characterization.

(5-(2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)picolinoyl)-l-glutamic acid (13c).

Compound 13c was prepared using the similar procedure to 28d, from 43d (0.52 g, 1.07 mmol) to give 13c (321 mg, 70%) as a brown powder. mp 225 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.61 (s, 1H, NH), 10.13 (s, 1H, NH), 8.79 (d, J = 8.1 Hz, 1H, NH), 8.52 (d, J = 2.0 Hz, 1H, Ar), 7.94 (d, J = 8.0 Hz, 1H, Ar), 7.83 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 6.35 (d, J = 2.0 Hz, 1H, Ar), 5.91 (s, 2H, 2-NH₂), 4.47 (td, J = 8.8, 4.8 Hz, 1H, CH), 2.71 (d, J = 7.3 Hz, 2H, CH₂), 2.63 – 2.57 (m, 2H, CH₂), 2.29 (t, J = 7.4 Hz, 2H, CH₂), 2.18 – 1.96 (m, 2H, CH₂). Anal. Calcd for C₂₁H₂₄N₆O₆·0.3949 H₂O: C, 54.41; H, 5.39; N, 18.13. Found: C, 54.35; H, 5.10; N, 18.21.

(5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)picolinoyl)-l-glutamic acid (15c).

Compound 15c was prepared using the similar procedure to 28d, from 44d (0.70 g, 1.36 mmol) to give 15c (392 mg, 63%) as a buff colored powder. mp: 170.2 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.60 (s, 1H, NH), 10.08 (s, 1H, NH), 8.81 (d, J = 8.3 Hz, 1H, NH), 8.48 (d, J = 2.1 Hz, 1H, Ar), 7.95 (d, J = 7.8 Hz, 1H, Ar), 7.85 (dd, J = 8.3, 2.4 Hz, 1H, Ar), 6.38 (d, J = 2.0 Hz, 1H, Ar), 5.94 (s, 2H), 4.49 (td, J = 8.1, 4.6 Hz, 1H, CH), 2.73 (d, J = 7.1 Hz, 2H, CH₂), 2.65 (m, 2H, CH₂), 2.31 (t, J = 7.7 Hz, 2H, CH₂), 2.23 – 1.85 (m, 2H, CH₂), 1.66 – 1.57 (m, 4H, CH₂). Anal. Calcd for C₂₁H₂₄N₆O₆·0.4455 H₂O: C, 54.30; H, 5.40; N, 18.09. Found: C, 54.23; H, 5.10; N, 18.21.
(5-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)picolinoyl)-L-glutamic acid (16c).

Compound 16c was prepared using the similar procedure to 28d, from 45d (0.49 g, 0.93 mmol) to give 16c (330 mg, 75%) as a brown colored powder. mp: 234 °C. $^1$H NMR (400 MHz, DMSO-$_d$6) $\delta$ 10.63 (s, 1H, NH), 10.14 (s, 1H, NH), 8.81 (d, J = 7.9 Hz, 1H, NH), 8.54 (d, J = 2.0 Hz, 1H, Ar), 7.91 (d, J = 8.3 Hz, 1H, Ar), 7.86 (dd, J = 7.5, 2.1 Hz, 1H, Ar), 6.33 (d, J = 1.9 Hz, 1H, Ar), 6.01 (s, 2H, 2-NH$_2$), 4.45 (td, J = 8.9, 4.1 Hz, 1H, CH), 2.69 (t, J = 7.6 Hz, 2H, CH$_2$), 2.55 (d, J = 7.8 Hz, 2H, CH$_2$), 2.30 (t, J = 7.5 Hz, 2H, CH$_2$), 2.20 – 1.93 (m, 2H, CH$_2$), 1.71 – 1.55 (m, 4H, CH$_2$), 1.37 – 1.25 (m, 2H, CH$_2$). Anal. Calcd for C$_{22}$H$_{26}$N$_6$O$_6$: C, 56.16; H, 5.57; N, 17.86. Found: C, 55.97; H, 5.35; N, 17.67.

(6-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)nicotinoyl)-L-glutamic acid (17c).

Compound 17c was prepared using the similar procedure to 28d, from 60d (0.25 g, 0.51 mmol) to give 17c (0.14 g, 64%) as a brown powder. mp: 181.5 °C. $^1$H NMR (400 MHz, DMSO-$_d$6) $\delta$ 10.67 (s, 1H, NH), 10.14 (s, 1H, NH), 8.92 (d, J = 8.0 Hz, 1H, NH), 8.05 (dd, J = 7.9, 2.0 Hz, 1H, Ar), 7.33 (d, J = 7.0 Hz, 1H, Ar), 7.28 (d, J = 8.1 Hz, 1H, Ar), 6.39 (d, J = 2.0 Hz, 1H, Ar), 6.01 (s, 2H, 2-NH$_2$), 4.51 (m, J = 9.5, 8.0 Hz, 1H, CH), 2.71 (t, J = 7.6 Hz, 2H, CH$_2$), 2.60 (t, J = 7.4 Hz, 2H, CH$_2$), 2.43 – 2.32 (m, 2H, CH$_2$), 2.28 – 2.13 (m, 2H, CH$_2$), 2.05 (q, J = 7.6 Hz, 2H, CH$_2$). Anal. Calcd for C$_{20}$H$_{22}$N$_6$O$_6$ · 0.5705 H$_2$O: C, 53.06; H, 5.15; N, 18.56. Found: C, 52.95; H, 4.77; N, 18.75.

(6-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)nicotinoyl)-L-glutamic acid (18c).

Compound 18c was prepared using the similar procedure to 28d, from 61d (0.42 g, 0.82 mmol) to give 18c (282 mg, 75%) as a brown powder. mp: 241.7 °C. $^1$H NMR (400 MHz, DMSO-$_d$6) $\delta$ 10.62 (s, 1H, NH), 10.13 (s, 1H, NH), 8.84 (d, J = 8.2 Hz, 1H, NH), 8.07 (dd, J = 8.1, 2.2 Hz,
1H, Ar), 7.32 (d, J = 7.1 Hz, 1H, Ar), 7.25 (d, J = 8.1 Hz, 1H, Ar), 6.35 (d, J = 2.0 Hz, 1H, Ar),
5.97 (s, 2H, 2-NH₂), 4.47 (td, J = 8.8, 4.8 Hz, 1H, CH), 2.74 (d, J = 7.3 Hz, 2H, CH₂), 2.60 –
2.55 (m, 2H, CH₂), 2.32 (t, J = 7.4 Hz, 2H, CH₂), 2.19 – 2.11 (m, 2H, CH₂), 1.69 – 1.58 (m,
4H, CH₂). Anal. Calcd for C₁₂₁H₂₄₅N₆O₆·0.4773 H₂O: C, 54.24; H, 5.41; N, 18.07. Found: C,
54.27; H, 5.12; N, 17.87.

(6-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)nicotinoyl)-
L-glutamic acid (19c).

Compound 19c was prepared using the similar procedure to 28d, from 62d (0.47 g, 0.89 mmol)
to give 19c (294 mg, 70%) as a reddish brown powder mp: 221.6 °C. ^1H NMR (400 MHz,
DMSO-d₆) δ 10.59 (s, 1H, NH), 10.11 (s, 1H, NH), 8.91 (d, J = 7.8 Hz, 1H, Ar), 8.09 (dd, J =
8.0, 1.9 Hz, 1H, Ar), 7.35 (d, J = 7.6 Hz, 1H, Ar), 7.26 (d, J = 8.1 Hz, 1H, Ar), 6.17 (d, J = 2.2
Hz, 1H, Ar), 5.89 (s, 2H, 2-NH₂), 4.53 (td, J = 8.5, 4.3 Hz, 1H, CH), 2.69 (t, J = 7.7 Hz, 2H,
CH₂), 2.52 (d, J = 7.6 Hz, 2H, CH₂), 2.30 (t, J = 7.3 Hz, 2H, CH₂), 2.20 – 1.98 (m, 2H, CH₂),
1.63 (m, 4H, CH₂), 1.36 – 1.25 (m, 2H, CH₂). Anal. Calcd for C₂₅H₂₄₅N₆O₆·0.5848 H₂O: C,
54.93; H, 5.69; N, 17.47. Found: C, 54.86; H, 5.51; N, 17.64.

General procedure for the synthesis of 67d-69d.

To a solution of triphenylphosphine (1 eq) in dry methylene chloride (100 mL) was
added iodine (1 eq) and imidazole (1 eq) at 0 °C. The resulting solution was stirred for 10 min,
before a solution of alcohols 74d-76d (1 eq.) in dry methylene chloride (50 mL) was added.
The reaction mixture was then quenched with sat. aqueous sodium thiosulfite solution after 30
minutes. The organic layer was separated and washed with brine and dried over Na₂SO₄. Silica
gel was then added, and the solvent was evaporated under reduced pressure. The resulting plug
was loaded on to a silica gel column with ethyl acetate-Hexane as the eluent. Fractions that
showed the desired spot (TLC) were pooled and the solvent evaporated to dryness to afford
corresponding iodides.
Methyl 5-(3-iodopropyl)picolinate (67d).

Compound 67d was prepared using above general procedure, from 74d (2.25 g, 11.53 mmol), iodine (4.39 g, 17.29 mmol), triphenyl phosphine (4.53 g, 17.29 mmol), and imidazole (1.18 g, 17.29 mmol) to afford 67d (3.01 g, 85%) as yellow oil. TLC R_f = 0.88 (Hexane: EtOAc, 3:1); 
1H NMR (400 MHz, DMSO-d_6) δ 8.59 (d, J = 2.0 Hz, 1H, Ar), 8.00 (d, J = 8.0 Hz, 1H, Ar), 7.89 – 7.83 (m, 1H, Ar), 3.87 (s, 3H, OCH_3), 3.26 (t, J = 6.8 Hz, 2H, CH_2), 2.83 – 2.72 (m, 2H, CH_2), 2.16 – 2.04 (m, 2H, CH_2). The intermediate 67d was used for the next reaction without further characterization.

Methyl 5-(4-iodobutyl)picolinate (68d).

Compound 68d was prepared using above general procedure, from 75d (2.49 g, 11.90 mmol), iodine (4.53 g, 17.85 mmol), triphenyl phosphine (4.68 g, 17.85 mmol), and imidazole (1.2 g, 17.85 mmol) to afford 68d (3.10 g, 82%) as yellow oil. TLC R_f = 0.84 (Hexane: EtOAc, 3:1); 
1H NMR (400 MHz, DMSO-d_6) δ 8.58 (m, 1H, Ar), 7.99 (dd, J = 8.0, 0.7 Hz, 1H, Ar), 7.86 – 7.80 (m, 1H, Ar), 3.87 (s, 3H, OCH_3), 3.29 (t, J = 6.9 Hz, 2H, CH_2), 2.68 (t, J = 7.6 Hz, 2H, CH_2), 1.81 – 1.75 (m, 2H, CH_2), 1.64 (p, J = 7.4 Hz, 2H, CH_2). The intermediate 68d was used for the next reaction without further characterization.

Methyl 5-(5-iodopentyl)picolinate (69d).

Compound 69d was prepared using above general procedure, from 76d (2.51 g, 11.15 mmol), iodine (4.25 g, 16.73 mmol), triphenyl phosphine (4.39 g, 16.73 mmol), and imidazole (1.14 g, 16.73 mmol) to afford 69d (2.99 g, 80%) as yellow oil. TLC R_f = 0.87 (Hexane: EtOAc, 3:1); 
1H NMR (400 MHz, DMSO-d_6) δ 8.61 – 8.56 (m, 1H, Ar), 7.97 (dd, J = 7.9, 0.9 Hz, 1H, Ar), 7.84 (m, 1H, Ar), 3.89 (s, 3H, OCH_3), 3.26 (t, J = 6.6 Hz, 2H, CH_2), 2.69 (t, J = 7.9 Hz, 2H, CH_2), 1.83 – 1.73 (m, 2H, CH_2), 1.62 (p, J = 7.6 Hz, 2H, CH_2), 1.37 (t, J = 6.8 Hz, 2H, CH_2). The intermediate 69d was used for the next reaction without further characterization.
2-Amino-6-methyl-5-nitro-4(3H)-oxopyrimidine (81d).

In a 100 mL, round-bottomed flask, 2-amino-6-methyl-4(3H)-oxopyrimidin 80d (20 g, 0.16 mmol) was dissolved in H$_2$SO$_4$ (100 mL) and cooled to 0 °C, and HNO$_3$ (17.6 mL) was added dropwise over 30 min. The mixture was warmed to rt, stirred for 3 h, and poured into Et$_2$O (1500 mL), and the precipitate was filtered and dissolved in 1 N NaOH. AcOH was added to precipitate product, and the solid was collected by vacuum filtration, washed with H$_2$O (2 x 10 mL), and air-dried overnight in a vacuum oven to yield 26.3 g (97%) of 81d as a pale yellow solid: mp > 300 °C, (lit. mp > 300 °C) $^1$H NMR (400 MHz, DMSO-$_d_6$) δ 11.62 (s, 1H, NH), 6.25 (s, 2H, 2-NH$_2$), 2.24 (s, 3H, CH$_3$).

$N'$-[4-[2-(Dimethylamino)ethenyl]-1,6-dihydro-5-nitro-6-oxo-2-pyrimidinyl]-N,N dimethylmethanimidamide (82d).

In a 100 mL two-neck round-bottomed flask under N$_2$ atmosphere and molecular sieve 3 Å was added 81d (2.0 g, 11.7 mmol) in dry DMF (25 mL) and DMF dimethyl acetal (7.5 mL, 70.0 mmol). The mixture was stirred at 100 °C for 24 h and then cooled. The mixture was dried under vacuum. Acetone (15 mL) was added and the formed precipitate was filtered and washed with acetone (10 mL) furnishing 82d in 90% yield (2.96 g) as an orange solid, mp: 275 °C (Lit.$^{351}$ 276-278 °C). $^1$H NMR (400 MHz, DMSO-$_d_6$) δ 8.58 (s, 1H, CH), 7.88 (d, J = 12.2 Hz, 1H, CH), 5.60 (d, J = 12.2 Hz, 1H, CH), 3.24 (s, 3H, CH$_3$), 3.14 (s, 3H, CH$_3$), 2.80–3.10 (m, 6H, CH$_3$).

2-Amino-3,5-dihydro-pyrrolo[3,2-d]pyrimidin-4-one (83d).

In a 500 mL round-bottomed flask were combined 82d (4.6 g, 16.4 mmol), sodium dithionite (12.0 g, 69 mmol) and THF/H$_2$O (2:1, 300 mL). The mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure, then H$_2$O (50 mL) was added and the solid formed was filtered and washed with water (2 x 100 mL). The product was purified by silica gel chromatography using 2% MeOH in CH$_2$Cl$_2$ as eluent. Compound 83d was
obtained in 89% yield (2.99 g) as a yellow solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 11.58 (s, 1H, NH), 8.51 (s, 1H, CH), 7.19 (t, $J = 5.9$ Hz, 1H, Ar), 6.09 (t, $J = 4.7$ Hz, 1H, Ar), 3.13 (s, 3H, CH$_3$), 3.03 (s, 3H, CH$_3$).

2-Amino-3,5-dihydro-4H-pyrrolo[3,2-$d$]pyrimidin-4-one (66d).

In a microwave vessel was added 83d (1 g, 4.87 mmol) and NaOH (975 mg, 24.36 mmol) in H$_2$O (10mL). The set conditions were 90 °C for 15 min in the Biotage® microwave. This procedure was repeated four times and then the crude products were mixed. The solvent was evaporated under vacuum and then water (5 mL) was added. The mixture was acidified to pH 5.0 with glacial acetic acid. The precipitate was affording the desired product 66d in 90% yield (660 mg) as a white solid, mp: decomposed at 300 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.43 (d, $J = 5.9$ Hz, 1H, Ar), 6.44 (s, 2H, 2-NH$_2$), 5.87 (d, $J = 4.9$ Hz, 1H, Ar).

$N$-[(4-Oxo-4,5-dihydro-3H-pyrrolo[3,2-$d$]pyrimidin-2-yl)pivalamide (84d).

To a round bottomed flask were added 66d (660 mg, 4.4 mmol) and pivalic anhydride (10 ml). The mixture was refluxed at 110 °C for 4 h. After 4 h, the reaction mixture was cooled in ice bath and 100 mL of hexanes was added to precipitate the compound 84d in 85% (880 mg) yield as a white solid. Mp: decomposed at 300 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.16 (s, 1H, NH), 10.08 (s, 1H, NH), 7.41 (t, $J = 2.9$ Hz, 1H, Ar), 6.36 – 6.32 (m, 1H, Ar), 1.24 (s, 9H, CH$_3$).

$N$,$N$-Dimethyl-$N'$-(4-methyl-5-nitro-6-oxo-1,6-dihydropyrimidin-2-yl)formimidamide (87d).

In a 50 mL round bottom flask flushed with nitrogen were combined 81d (4.0 g, 23.6 mmol), DMF dimethylacetal (20 mL), and CH$_2$Cl$_2$ (30 mL). The reaction mixture was stirred at rt for 4 h, the solvent was removed under reduced pressure, the crude product was purified by column chromatography on silica gel with 0.5-1% MeOH:CH$_2$Cl$_2$ as the eluent to yield 3.2 g (60%) of 87d as an orange solid. TLC $R_f = 0.38$ (CHCl$_3$:MeOH, 10:1); mp: 207-209 °C; $^1$H NMR
(400 MHz, DMSO-\textit{d}_6) \delta 12.27 (s, 1H, NH), 8.73 (s, 1H, CH), 3.22 (s, 3H, CH$_3$), 3.09 (s, 3H, CH$_3$), 2.28 (s, 3H, CH$_3$).

(2-(((Dimethylamino)methylene)amino)-4-methyl-5-nitro-6-oxopyrimidin-1(6H)-yl)methyl pivalate (89d), ((2-(((dimethylamino)methylene)amino)-6-methyl-5-nitropyrimidin-4-yl)oxy)methyl pivalate (90d).

In a two-neck 100 mL round-bottomed flask flushed with argon were placed NaH (0.46 g, 11.5 mmol, 60% in mineral oil) and DMF (anhyd) 20 mL. To this mixture was added 87d (2.35 g, 10.4 mmol) slowly. The mixture became viscous and could stir at rt for 1 h. Chloromethyl pivalate (2 mL) was added dropwise over 5 min, and the reaction was stirred for 12 h at rt, after which time it became fluid. Solvent was removed under reduced pressure, the residue was dissolved in CH$_2$Cl$_2$ (50 mL), extracted with 5% AcOH (1 x 30 mL) and H$_2$O (1 x 30 mL), dried with MgSO$_4$, and filtered, and the solvent was removed under reduced pressure. The crude products were purified by column chromatography on silica gel with CH$_2$Cl$_2$-0.5% MeOH:CH$_2$Cl$_2$ as the eluent to yield 2.27 g (65%) of 89d as a pale yellow solid: mp 134-136 °C; $^1$H NMR (400 MHz, Chloroform-\textit{d}) $\delta$ 8.73 (s, 1H, CH), 6.21 (s, 2H, CH$_2$), 3.26 (s, 3H, CH$_3$), 3.14 (s, 3H, CH$_3$), 2.37 (s, 3H, CH$_3$), 1.15 (s, 9H, CH$_3$), and 0.53 g (15%) of 90d as a pale yellow gum: $^1$H NMR (400 MHz, Chloroform-\textit{d}) $\delta$ 8.75 (s, 1H, CH), 6.17 (s, 2H, CH$_2$), 3.23 (s, 3H, CH$_3$), 3.21 (s, 3H, CH$_3$), 2.52 (s, 3H, CH$_3$), 1.17 (s, 9H, CH$_3$).


In a 50-mL round-bottomed flask flushed with nitrogen were combined 90d (2.27 g, 6.6 mmol), DMF (anhyd) (20 mL), and DMF dimethylacetal (5 mL). The reaction mixture was stirred for 12 h at rt, the solvent was removed under reduced pressure, 20% ether/hexanes was added, and the solid was filtered and dried under house vacuum. The product was purified by column chromatography on silica gel with CH$_2$Cl$_2$-0.5% MeOH:CH$_2$Cl$_2$ as the eluent to yield 2.48 g
(90%) of 91d as yellowish white solid. TLC \( R_f = 0.85 \) (CHCl₃:MeOH, 10:1); mp: 206-207 °C;
\(^1\)H NMR (400 MHz, Chloroform-\(d\)) \( \delta \) 8.63 – 8.57 (m, 1H, CH), 7.99 (d, \( J = 12.2 \) Hz, 1H, CH), 6.18 (s, 2H, CH₂), 5.74 (d, \( J = 12.2 \) Hz, 1H, CH), 3.25 (s, 3H, CH₃), 3.18 (s, 3H, CH₃), 3.11 (s, 3H, CH₃), 2.98 – 2.89 (s, 3H, CH₃).

2-[(N,N-Dimethylamino)methyleneamino]-4(3\(H\))-oxo-3-[(pivaloyloxy)methyl]-5\(H\)-pyrrolo[3,2-\(d\)]pyrimidine (92d).

In a 100 mL round-bottomed flask were combined 91d (2 g, 5.07 mmol), Na₂S₂O₄ (5.30 g, 30.42 mmol), and THF:H₂O (2:1, 30 mL). The mixture was stirred at rt for 1 h, the solvent was removed under reduced pressure, H₂O (10 mL) was added, and the solid was collected by vacuum filtration and washed with H₂O (2 x 10 mL) to yield 1.44 g (89%) of 92d as a white powder: mp 266.4 °C; \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \( \delta \) 11.82 (s, 1H, NH), 8.56 (s, 1H, CH), 7.30 (s, 1H, Ar), 6.20 (s, 2H, CH₂), 6.14 (s, 1H, Ar), 3.14 (s, 3H, CH₃), 2.97 (s, 3H, CH₃), 1.10 (s, 9H, CH₃).

General procedure for the synthesis of 93d-95d.

In a two-neck 100 mL round-bottomed flask flushed with argon were placed NaH (1.5 eq) and DMF(anhyd). To this mixture was added 92d (1 eq.) slowly. The mixture was allowed to stir at rt for 2 h and solution of iodides 67d-69d in DMF (5 mL) were added dropwise over 10 min. The mixture was stirred for 2 h, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography on silica gel with 3% MeOH:CH₂Cl₂ as the eluent to yield 93-95d as sticky oil.

Methyl 5-(3-(2-(((dimethylamino)methylene)amino)-4-oxo-3-((pivaloyloxy)methyl)-3,4-dihydro-5\(H\)-pyrrolo[3,2-\(d\)]pyrimidin-5-yl)propyl)picolinate (93d).

Compound 93d was prepared using the above general procedure from 92d (0.7 g, 2.19 mmol), NaH (87.7 mg, 2.19 mmol) and 67d (1 g, 3.29 mmol) to afford 93d (550 mg, 50%) as a yellow oil. TLC \( R_f = 0.79 \) (CHCl₃:MeOH, 10:1); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 8.59 (d, \( J = 2.2 \))
Hz, 1H, Ar), 8.53 (s, 1H, CH), 7.98 – 7.94 (m, 1H, Ar), 7.85 (dd, J = 8.2, 2.2 Hz, 1H, Ar), 7.41 (d, J = 3.1 Hz, 1H, Ar), 6.22 (s, 2H, CH₂), 6.18 – 6.15 (m, 1H, Ar), 4.25 – 4.16 (m, 2H, CH₂), 3.87 (s, 3H, CH₃), 3.14 (s, 3H, CH₃), 2.85 (s, 3H, CH₃), 2.75 (q, J = 8.2, 8.1 Hz, 2H, CH₂), 1.98 – 1.91 (m, 2H, CH₂), 1.08 (s, 9H, CH₃). The intermediate 93d was used for the next reaction without further characterization.

Methyl 5-(4-(2-(((dimethylamino)methylene)amino)-4-oxo-3-((pivaloyloxy)methyl)-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinate (94d).

Compound 94d was prepared using the above general procedure from 92d (1g, 3.13 mmol), NaH (125 mg, 3.13 mmol) and 68d (1.5 g, 4.7 mmol) to afford 94d (644 mg, 40%) as a yellow oil. TLC Rf = 0.83 (CHCl₃:MeOH, 10:1); ¹H NMR (400 MHz, DMSO-d₆) δ 8.58 (d, J = 1.8 Hz, 1H, Ar), 8.55 (s, 1H, CH), 7.93 (d, J = 7.8 Hz, 1H, Ar), 7.81 (d, J = 1.7 Hz, 1H, Ar), 7.21 (d, J = 0.3 Hz, 1H, Ar), 6.22 (s, 2H, CH₂), 5.88 (d, J = 2.6 Hz, 1H, Ar), 4.26 (t, J = 6.7 Hz, 2H, CH₂), 3.85 (s, 3H, CH₃), 3.56 (t, J = 4.7 Hz, 2H, CH₂), 3.17 (s, 3H, CH₃), 1.74 (t, J = 7.2 Hz, 2H, CH₂), 1.51 (m, 2H, CH₂), 1.08 (s, 9H, CH₃). The intermediate 94d was used for the next reaction without further characterization.

Methyl 5-(5-(2-(((dimethylamino)methylene)amino)-4-oxo-3-((pivaloyloxy)methyl)-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)picolinate (95d).

Compound 95d was prepared using the above general procedure from 92d (0.85 g, 2.66 mmol), NaH (106 mg, 2.66 mmol) and 68d (1.33 g, 3.99 mmol) to afford 95d (671 mg, 48%) as a yellow oil. TLC Rf = 0.80 (CHCl₃:MeOH, 10:1); ¹H NMR (400 MHz, DMSO-d₆) δ 8.57 (s, 1H, CH), 8.54 (d, J = 2.1 Hz, 1H, Ar), 7.96 (d, J = 8.0 Hz, 1H, Ar), 7.79 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 7.39 (d, J = 3.1 Hz, 1H, Ar), 6.24 (s, 2H, CH₂), 6.17 (d, J = 3.1 Hz, 1H, Ar), 4.22 – 4.14 (m, 2H, CH₂), 3.87 (s, 3H, CH₃), 3.16 (s, 2H, CH₂), 2.99 (s, 3H, CH₃), 2.71 – 2.61 (m, 2H, CH₂), 1.71 – 1.56 (m, 4H, CH₂), 1.33 (s, 2H, CH₂), 1.07 (s, 9H, CH₃). The intermediate 95d was used for the next reaction without further characterization.
5-(3-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)propyl)picolinic acid (96d).

To a 25 mL round-bottomed flask was added 93d (0.55 g, 1.11 mmol), 1N NaOH (9.97 mL, 9.97 mmol) and MeOH (5 mL), stirred overnight. MeOH was evaporated and reaction was acidified to pH 5 using 1N HCl to afford 96d (244 mg, 70%) as a white precipitate.

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.51 (d, J = 2.0 Hz, 1H, Ar), 7.94 (d, J = 7.9 Hz, 1H, Ar), 7.84 – 7.74 (m, 1H, Ar), 7.22 (d, J = 2.9 Hz, 1H, Ar), 5.90 (d, J = 2.8 Hz, 1H, Ar), 5.83 (s, 2H, 2-NH$_2$), 4.27 (t, J = 6.9 Hz, 2H, CH$_2$), 2.62 (t, J = 7.8 Hz, 2H, CH$_2$), 2.09 (t, J = 7.5 Hz, 2H, CH$_2$). The intermediate 96d was used for the next reaction without further characterization.

5-(4-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinic acid (97d).

Compound 97d was prepared using similar procedure to 96d from 94d (644 mg, 1.26 mmol) to afford 226 mg of target compound as a buff colored precipitate in 55% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.49 (d, J = 1.9 Hz, 1H, Ar), 7.93 (d, J = 8.0 Hz, 1H, Ar), 7.73 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 7.20 (d, J = 2.8 Hz, 1H, Ar), 5.88 (d, J = 2.7 Hz, 1H, Ar), 5.82 (s, 2H, 2-NH$_2$), 4.26 (t, J = 6.9 Hz, 2H, CH$_2$), 2.66 (t, J = 7.7 Hz, 2H, CH$_2$), 1.74 (p, J = 6.9 Hz, 2H, CH$_2$), 1.51 (q, J = 7.8 Hz, 2H, CH$_2$). Mass calculated for C$_{16}$H$_{17}$N$_3$O$_3$ [M + H]$^+$, 328.14 Found 328.4. The intermediate 97d was used for the next reaction without further characterization.

5-(5-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)picolinic acid (98d).

Compound 98d was prepared using similar procedure to 96d from 95d (671 mg, 1.72 mmol) to afford 381 mg of target compound as a white precipitate in 65% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.54 (d, J = 2.2 Hz, 1H, Ar), 7.95 (d, J = 7.9 Hz, 1H, Ar), 7.78 (dd, J = 8.1, 2.2 Hz, 1H, Ar), 7.17 (d, J = 2.8 Hz, 1H, Ar), 5.87 (d, J = 2.8 Hz, 1H, Ar), 5.74 (s, 2H, 2-NH$_2$), 4.21 (t, J = 7.0 Hz, 2H, CH$_2$), 2.72 – 2.60 (m, 2H, CH$_2$), 1.79 – 1.69 (m, 2H, CH$_2$), 1.68 – 1.54
(m, 2H, CH₂), 1.23 (m, 2H, CH₂). Mass calculated for C₁₇H₁₉N₅O₃ [M - H]⁺, 340.14 Found 340.5. The intermediate 98d was used for the next reaction without further characterization.

Diethyl (5-(3-(2-amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)propyl)picolinoyl)-L-glutamate (99d).

Compound 99d (245 mg, 63%) was prepared as a brown syrup using similar glutamate coupling conditions as 29d, from 96d (244 mg, 0.78 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (164 mg, 0.93 mmol), 4-methylmorpholine (0.1 mL, 0.93 mmol) and diethyl L-glutamate HCl (237 mg, 1.17 mmol). TLC Rf = 0.51 (CHCl₃:MeOH, 10:1). ¹H NMR (400 MHz, DMSO-d₆) δ 8.92 (d, J = 8.2 Hz, 1H, NH), 8.51 (d, J = 2.2 Hz, 1H, Ar), 7.93 (d, J = 8.0 Hz, 1H, Ar), 7.83 (d, J = 8.0 Hz, 1H, Ar), 5.95 (s, 2H, 2-NH₂), 5.93 (d, J = 2.8 Hz, 1H, Ar), 4.52 (m, 1H, CH), 4.26 (t, J = 6.9 Hz, 2H, CH₂), 4.21 (q, J = 7.1 Hz, 2H, CH₃), 4.16–4.10 (m, 2H, CH₂), 2.65 (t, J = 7.8 Hz, 2H, CH₂), 2.43–2.31 (m, 2H, CH₂), 2.21–1.98 (m, 4H, CH₂), 1.25 (t, J = 7.1 Hz, 3H, CH₃), 1.14 (t, J = 7.1 Hz, 3H, CH₃). The intermediate 99d was used for the next reaction without further characterization.

Diethyl (5-(4-(2-amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinoyl)-l-glutamate (100d).

Compound 100d (206 mg, 58%) was prepared as a yellow syrup using similar glutamate coupling conditions as 29d, from 97d (226 mg, 0.67 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (145 mg, 0.83 mmol), 4-methylmorpholine (0.09 mL, 0.83 mmol) and diethyl L-glutamate HCl (211 mg, 1.04 mmol). TLC Rf = 0.54 (CHCl₃:MeOH, 10:1). ¹H NMR (400 MHz, DMSO-d₆) δ 8.90 (d, J = 8.2 Hz, 1H, NH), 8.50 (d, J = 1.9 Hz, 1H, Ar), 7.93 (d, J = 8.0 Hz, 1H, Ar), 7.83–7.77 (m, 1H, Ar), 7.21 (d, J = 2.9 Hz, 1H, Ar), 5.88 (d, J = 2.7 Hz, 1H, Ar), 5.74 (s, 2H, 2-NH₂), 4.53 (m, 1H, CH), 4.26 (t, J = 6.8 Hz, 2H, CH₂), 4.11 (q, J = 5.2 Hz, 2H, CH₂), 4.01 (q, J = 7.1 Hz, 2H, CH₂), 3.56 (t, J = 4.7 Hz, 2H, CH₂), 2.69 (t, J = 7.8 Hz, 2H, CH₂), 2.37 (dd, J = 8.1, 5.8 Hz, 2H, CH₂), 1.78–1.70 (m, 2H, CH₂), 1.52 (d, J = 8.0 Hz, 2H,
CH_{2}), 1.19 (t, J = 7.2 Hz, 3H, CH_{3}), 1.15 (t, J = 7.2 Hz, 3H, CH_{3}). Mass calculated for C_{25}H_{32}N_{6}O_{6} [M + H]^+, 513.24 Found 513.5. The intermediate 100d was used for the next reaction without further characterization.

Diethyl (5-(5-(2-amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)picolinoyl)-l-glutamate (101d).

Compound 101d (386 mg, 66%) was prepared as a brown syrup using similar glutamate coupling conditions as 29d, from 98d (381 mg, 1.12 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (235 mg, 1.34 mmol), 4-methylmorpholine (0.15 mL, 1.34 mmol) and diethyl l-glutamate HCl (340 mg, 1.67 mmol). TLC R_{f} = 0.52 (CHCl_{3}:MeOH, 10:1). \^{1}H NMR (400 MHz, DMSO-d_{6}) \delta 8.90 (d, J = 8.2 Hz, 1H, NH), 8.51 (d, J = 2.1 Hz, 1H, Ar), 7.93 (d, J = 8.0 Hz, 1H, Ar), 7.81 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 7.16 (d, J = 2.8 Hz, 1H, Ar), 5.87 (d, J = 2.8 Hz, 1H, Ar), 5.73 (s, 2H, 2-NH_{2}), 4.53 (td, J = 8.8, 5.1 Hz, 1H, CH), 4.21 (t, J = 6.9 Hz, 2H, CH_{2}), 4.15 – 4.08 (m, 2H, CH_{2}), 4.03 (p, J = 7.1 Hz, 2H, CH_{2}), 2.67 (q, J = 9.4, 7.5 Hz, 2H, CH_{2}), 2.41 – 2.32 (m, 2H, CH_{2}), 2.25 – 2.01 (m, 2H, CH_{2}), 1.85 – 1.70 (m, 2H, CH_{2}), 1.59 (dd, J = 15.5, 7.7 Hz, 2H, CH_{2}), 1.24 – 1.22 (m, 2H, CH_{2}), 1.19 (t, J = 7.1 Hz, 3H, CH_{3}), 1.14 (t, J = 7.1 Hz, 3H, CH_{3}). Mass calculated for C_{26}H_{34}N_{6}O_{6} [M + H]^+, 527.26 Found 527.4. The intermediate 101d was used for the next reaction without further characterization.

(5-(3-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)propyl)picolinoyl)-l-glutamic acid (20c).

Compound 20c (383 mg, 88%) was prepared using the similar procedure to 28d, from 99d (0.49 g, 0.98 mmol) as a brown colored powder. mp 206.1 °C. \^{1}H NMR (400 MHz, DMSO-d_{6}) \delta 8.79 (d, J = 8.1 Hz, 1H, NH), 8.50 (d, J = 2.1 Hz, 1H, Ar), 7.94 (d, J = 8.0 Hz, 1H, Ar), 7.83 (dd, J = 8.1, 2.1 Hz, 1H, Ar), 7.22 (d, J = 2.9 Hz, 1H, Ar), 5.94 – 5.86 (m, 1H, Ar), 5.80 (s, 2H, 2-NH_{2}), 4.48 (td, J = 8.7, 4.9 Hz, 1H, CH), 4.28 (t, J = 7.0 Hz, 2H, CH_{2}), 2.64 (t, J = 7.8 Hz, 2H, CH_{2}), 2.29 (t, J = 7.3 Hz, 2H, CH_{2}), 2.19 – 1.96 (m, 4H, CH_{2}). Mass calculated for
C20H22N6O6 [M + Na]+, 465.16; Found 465.7. HPLC analysis: retention time, 12.75 min; peak area, 95.23 %; eluent A, H2O with 0.1% TFA: eluent B, ACN with 0.1% TFA; gradient elution (95% H2O to 10% H2O) over 45 min with flow rate of 0.5 mL/min and detection at 245 nm; column temperature, rt.

(5-(4-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamic acid (21c).

Compound 21c (136 mg, 74%) was prepared using the similar procedure to 28d, from 100d (0.21 g, 0.4 mmol) as a brown colored powder. mp 152.1 °C. 1H NMR (400 MHz, DMSO-d6) δ 8.77 (d, J = 7.7 Hz, 1H, NH), 8.48 (d, J = 2.1 Hz, 1H, Ar), 7.93 (d, J = 7.8 Hz, 1H, Ar), 7.79 (dd, J = 8.1, 2.2 Hz, 1H, Ar), 7.20 (d, J = 2.8 Hz, 1H, Ar), 5.88 (d, J = 2.8 Hz, 1H, Ar), 5.77 (s, 2H, 2-NH2), 4.43 – 4.35 (m, 1H, CH), 4.26 (t, J = 6.7 Hz, 2H, CH2), 2.68 (t, J = 7.7 Hz, 2H, CH2), 2.36 – 2.20 (m, 2H, CH2), 2.03 (q, J = 7.2 Hz, 2H, CH2), 1.73 (q, J = 7.1 Hz, 2H, CH2), 1.57 – 1.45 (m, 2H, CH2). Mass calculated for C21H24N6O6 [M + H]+, 457.18; Found 457.3. HPLC analysis: retention time, 7.93 min; peak area, 97.28 %; eluent A, H2O with 0.1% TFA: eluent B, ACN with 0.1% TFA; gradient elution (95% H2O to 0 % H2O) over 17 min with flow rate of 0.3 mL/min and detection at 245 nm; column temperature, rt.

(5-(5-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)picolinoyl)-L-glutamic acid (22c).

Compound 22c (311 mg, 90%) was prepared using the similar procedure to 28d, from 101d (0.39 g, 0.73 mmol) as a brown colored powder. mp 158.0 °C. 1H NMR (400 MHz, DMSO-d6) δ 8.78 (d, J = 7.8 Hz, 1H, NH), 8.49 (d, J = 2.1 Hz, 1H, Ar), 7.93 (d, J = 7.9 Hz, 1H, Ar), 7.80 (dd, J = 8.0, 2.1 Hz, 1H, Ar), 7.17 (d, J = 2.8 Hz, 1H, Ar), 5.86 (d, J = 2.8 Hz, 1H, Ar), 5.78 (s, 2H, 2-NH2), 4.39 (d, J = 7.3 Hz, 1H, CH), 4.21 (t, J = 6.9 Hz, 2H, CH2), 2.65 (t, J = 7.7 Hz, 2H, CH2), 2.36 – 2.25 (m, 2H, CH2), 2.08 – 1.98 (m, 2H, CH2), 1.74 (t, J = 7.4 Hz, 2H, CH2), 1.64 – 1.55 (m, 2H, CH2), 1.21 (m, 2H, CH2). Mass calculated for C22H26N6O6 [M + H]+,
471.19; Found 471.3. HPLC analysis: retention time, 8.07 min; peak area, 98.53 %; eluent A, 
H2O with 0.1% TFA: eluent B, ACN with 0.1% TFA; gradient elution (95% H2O to 0 % H2O) 
over 17 min with flow rate of 0.3 mL/min and detection at 245 nm; column temperature, rt.
Diethyl (5-bromo-3-fluoropicolinoyl)-L-glutamate (103d).

Diethyl L-glutamate HCl (2.54 g, 12.50 mmol) was dissolved in DMF (20 mL). In a separate 
flask, 5-bromo-3-fluoropicolinic acid (2.50 g, 11.36 mmol) was dissolved in DMF (20 mL).
The solution of diethyl L-glutamate HCl in DMF was then added, followed by DIPEA (5.94 
ml, 34.09 mmol) and HATU (4.32 g, 11.36 mmol). The mixture was stirred overnight at room 
temperature, then diluted with EtOAc (200 mL). The organic layer was washed sequentially 
with 50 mL of half saturated sodium chloride, 50 mL 10% citric acid (aq.), 50 mL of half 
saturated sodium chloride, 50 mL of saturated sodium bicarbonate (aq.), 50 mL water and twice 
with 50 mL brine. The organic layer was dried over sodium sulfate, filtered and concentrated 
under reduced pressure. Purification by column chromatography eluting 0-50% 
EtOAc/Hexanes gave the desired product 103d as a clear liquid (3.70 g, 80%). 

\( ^1H \text{ NMR} \) (400 MHz, Chloroform-d) \( \delta \) 8.51 (d, \( J = 1.7 \) Hz, 1H, Ar), 8.27 (d, \( J = 8.3 \) Hz, 1H, NH), 7.81 – 7.75 
(m, 1H, Ar), 4.81 (ddd, \( J = 8.0, 5.4, 2.7 \) Hz, 1H, CH), 4.26 (q, \( J = 7.1, 2.2 \) Hz, 2H, CH\(_2\)), 4.14 
(q, \( J = 7.1, 4.1 \) Hz, 2H, CH\(_2\)), 2.55 – 2.30 (m, 4H, CH\(_2\)), 1.35 – 1.30 (t, \( J = 7.1 \) Hz, 3H, CH\(_3\)), 
1.28 – 1.22 (t, \( J = 7.1 \) Hz, 3H, CH\(_3\)). The intermediate 103d was used for the next reaction 
without further characterization.

Diethyl (3-fluoro-5-(4-hydroxybut-1-yn-1-yl)picolinoyl)-L-glutamate (104d).

Compound 104d (2.58 g, 69%) was prepared as a brown oil using similar procedure to 23d, 
from 103d (3.7 g, 9.13 mmol) in anhydrous acetonitrile was added palladium chloride (65 mg, 
0.37 mmol), triphenylphosphate (96 mg, 0.37 mmol), copper iodide (278 mg, 1.46 mmol), 
triethylamine (12.73 mL, 91.3 mmol), and but-3-yn-1-ol (0.83 mL, 10.96 mmol). TLC \( R_f \) = 
0.54 (Hexane: EtOAc, 3:1) \( ^1H \text{ NMR} \) (400 MHz, DMSO-\( d_6 \)) \( \delta \) 8.53 (d, \( J = 1.8 \) Hz, 1H, Ar), 8.21
(d, J = 8.1 Hz, 1H, NH), 7.79 – 7.76 (m, 1H, Ar), 4.79 (m, 1H, Ar), 4.26 (q, J = 7.2 Hz, 2H, CH₂), 4.13 (q, J = 7.1 Hz, 2H, CH₂), 3.65 (dd, J = 6.4, 5.5 Hz, 2H, CH₂), 2.55 – 2.53 (m, 2H, CH₂), 2.58 – 2.42 (m, 2H, CH₂), 2.39 – 2.09 (m, 2H, CH₂), 1.33 (t, J = 7.1 Hz, 3H, CH₃), 1.26 (t, J = 7.1 Hz, 3H, CH₃). Mass calculated for C₁₉H₂₃FN₂O₆ [M + Na]+, 417.14; Found, 417.3.

The intermediate 104d was used for the next reaction without further characterization.

Diethyl (3-fluoro-5-(5-hydroxypent-1-yn-1-yl)picolinoyl)-L-glutamate (105d).

Compound 105d (2.42 g, 65%) was prepared as a yellowish brown oil using similar procedure to 23d, from 103d (3.7 g, 9.13 mmol) in anhydrous acetonitrile was added palladium chloride (65 mg, 0.37 mmol), triphenylphosphine (96 mg, 0.37 mmol), copper iodide (278 mg, 1.46 mmol), triethylamine (12.73 mL, 91.3 mmol), and but-3-yn-1-ol (0.99 mL, 10.96 mmol). TLC Rf = 0.52 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 8.52 (d, J = 1.7 Hz, 1H, Ar), 8.25 (d, J = 8.3 Hz, 1H, NH), 7.79 – 7.73 (m, 1H, Ar), 4.85 (m, J = 8.0, 5.4, 2.7 Hz, 1H, CH), 4.25 (q, J = 7.1, 2.2 Hz, 2H, CH₂), 4.15 (q, J = 7.1, 4.1 Hz, 2H, CH₂), 3.82 (t, J = 6.1 Hz, 2H, CH₂), 2.77 (t, J = 7.7 Hz, 2H, CH₂), 2.58 – 2.42 (m, 2H, CH₂), 2.39 – 2.09 (m, 2H, CH₂), 1.32 (t, J = 7.1 Hz, 3H, CH₃), 1.25 (t, J = 7.1 Hz, 3H, CH₃).

The intermediate 105d was used for the next reaction without further characterization.

Diethyl (3-fluoro-5-(4-hydroxybutyl)picolinoyl)-L-glutamate (106d).

Compound 106d (2.3 g, 88%) was prepared as a clear oil using similar catalytic hydrogenation procedure to 22d (condition B) from 104d (2.58 g, 6.54 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC Rf = 0.47 (Hexane: EtOAc, 3:1) ¹H NMR (400 MHz, Chloroform-d) δ 8.35 (d, J = 8.1 Hz, 1H, Ar), 8.27 (d, J = 8.2 Hz, 1H), 7.38 (dd, J = 11.5, 1.7 Hz, 1H, Ar), 4.84 (m, J = 8.4, 4.6 Hz, 1H, CH), 4.26 (q, J = 7.3 Hz, 2H, CH₂), 4.16 (q, J = 7.4 Hz, 2H, CH₂), 3.71 (q, J = 7.5 Hz, 2H, CH₂), 2.77 (t, J = 7.7 Hz, 2H, CH₂), 2.58 – 2.42 (m, 2H, CH₂), 2.39 – 2.09 (m, 2H, CH₂), 1.85 – 1.72 (m, 2H, CH₂), 1.70 – 1.62 (m, 2H, CH₂), 1.33 (t, J = 7.2 Hz, 3H, CH₃), 1.24 (t, J = 7.2 Hz, 3H, CH₃).
Mass calculated for $\text{C}_{19}\text{H}_{27}\text{FN}_{2}\text{O}_6 \left[ \text{M + H} \right]^+$, 399.19; Found, 399.5. The intermediate $106d$ was used for the next reaction without further characterization.

Diethyl (3-fluoro-5-(5-hydroxypentyl)picolinoyl)-L-glutamate ($107d$).

Compound $107d$ (1.94 g, 79%) was prepared as a clear oil using similar catalytic hydrogenation procedure to $22d$ (condition B) from $105d$ (2.42 g, 5.93 mmol), 10% palladium on activated carbon (2 g) in a Parr flask, in ethanol/MeOH (150 mL) at 55 psi for 5 h. TLC $R_f = 0.44$ (Hexane: EtOAc, 3:1) $^1$$H$ NMR (400 MHz, Chloroform-$d$) $\delta$ 8.34 (d, $J = 8.3$ Hz, 1H, Ar), 8.27 (d, $J = 7.9$ Hz, 1H, NH), 7.39 (dd, $J = 11.2$, 2.1 Hz, 1H, Ar), 4.85 (m, 1H, CH), 4.26 (q, $J = 6.9$ Hz, 2H, CH$_2$), 4.13 (q, $J = 7.1$ Hz, 2H, CH$_2$), 3.69 (q, $J = 7.5$ Hz, 2H, CH$_2$), 2.79 (t, $J = 7.5$ Hz, 2H, CH$_2$), 2.56 – 2.41 (m, 2H, CH$_2$), 2.37 – 2.10 (m, 2H, CH$_2$), 1.85 – 1.72 (m, 2H, CH$_2$), 1.70 – 1.62 (m, 2H, CH$_2$), 1.55 (m, 2H, CH$_2$), 1.32 (t, $J = 7.2$ Hz, 3H, CH$_3$), 1.26 (t, $J = 7.2$ Hz, 3H, CH$_3$). The intermediate $107d$ was used for the next reaction without further characterization.

**General procedure for synthesis of 108d-109d and 110d-111d.**

To the alcohols $106d$-$107d$, was added triethylamine (1.5 equivalent) and dichloromethane (25 mL). The reaction was cooled to 0 °C and purged with nitrogen gas. Under anhydrous conditions, methanesulfonyl chloride (1.50 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 semi-solid products $108d$-$109d$. To the intermediates ($108d$-$109d$) in acetone, sodium iodide (1 equivalent) was added and refluxed for 8 hours. The reaction mixture was filtered. The filtrate was evaporated to obtain $110d$-$111d$ as yellow oil.

Diethyl (3-fluoro-5-(4-iodobutyl)picolinoyl)-L-glutamate ($110d$).

Compound $110d$ was prepared using the general method described for the preparation of $108d$-$109d$ and $110d$-$111d$, from $106d$ (2.3 g, 5.7 mmol), methanesulfonyl chloride (0.67 mL, 8.66
mmol) and triethylamine (1.22 mL, 8.66 mmol) to form the intermediate 108d in 82% (2.25 g) yield. To this sodium iodide was added and the procedure was followed to give 1.3 g (82%) of 110d as a clear oil; TLC R\(_f\) = 0.63 (EtOAc:Hexane, 1:2); \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 8.35 (d, J = 8.4 Hz, 1H, Ar), 8.27 (d, J = 8.1 Hz, 1, NH), 7.42 – 7.35 (m, 1H, Ar), 4.83 (td, J = 8.2, 4.9 Hz, 1H, CH), 4.26 (qd, J = 7.1, 2.5 Hz, 2H, CH\(_2\)), 4.13 (q, J = 7.1 Hz, 2H, CH\(_2\)), 3.23 (t, J = 6.6 Hz, 2H, CH\(_2\)), 2.75 (t, J = 7.5 Hz, 2H, CH\(_2\)), 2.58 – 2.39 (m, 2H, CH\(_2\)), 2.39 – 2.10 (m, 2H, CH\(_2\)), 1.94 – 1.74 (m, 4H, CH\(_2\)), 1.33 (t, J = 7.2 Hz, 3H, CH\(_3\)), 1.25 (t, J = 7.1 Hz, 3H, CH\(_3\)). The intermediate 110d was used for the next reaction without further characterization.

Diethyl (3-fluoro-5-(5-iodopentyl)picolinoyl)-L-glutamate (111d).

Compound 111d was prepared using the general method described for the preparation of 108d-109d and 110d-111d, from 107d (1.94 g, 4.7 mmol), methanesulfonyl chloride (0.55 mL, 7.06 mmol) and triethylamine (0.99 mL, 7.06 mmol) to form the intermediate 109d in 82% (2.25 g) yield. To this sodium iodide was added and the procedure was followed to give 1.98 g (86%) of 111d as a clear oil; TLC R\(_f\) = 0.62 (EtOAc:Hexane, 1:2); \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 8.34 (d, J = 8.4 Hz, 1H), 8.29 (d, J = 7.9 Hz, 1H, NH), 7.37 (m, 1H, Ar), 4.75 (td, J = 7.7, 1H, CH), 4.24 (q, J = 7.1 Hz, 2H, CH\(_2\)), 4.17 (q, J = 7.3 Hz, 2H, CH\(_2\)), 3.28 (t, J = 6.6 Hz, 2H, CH\(_2\)), 3.11 (m, 2H, CH\(_2\)), 2.74 (t, J = 7.4 Hz, 2H, CH\(_2\)), 2.47 (m, 2H, CH\(_2\)), 2.34 – 2.15 (m, 2H, CH\(_2\)), 1.91-1.78 (m, 4H), 1.31 (t, J = 7.5 Hz, 3H, CH\(_3\)), 1.28 (t, J = 7.3 Hz, 3H, CH\(_3\)). The intermediate 111d was used for the next reaction without further characterization.

Diethyl (5-(4-((2-(((dimethylamino)methylene)amino)-4-oxo-3-((pivaloyloxy)methyl)-3,4-dihydro-5H-pyrrolo[3,2-\(d\)]pyrimidin-5-yl)butyl)-3-fluoropicolinoyl)-L-glutamate (112d).

In a two-neck 50 mL round-bottomed flask flushed with argon were placed NaH (13.78 mg, 0.34 mmol) and DMF(anhyd) 25 mL. To this mixture was added 92d (0.1 g, 0.31 mmol) slowly. The mixture was allowed to stir at rt for 2 h and solution of iodide 110d (0.24 g, 0.47 mmol) in DMF (5 mL) were added dropwise over 10 min. The mixture was stirred for 2 h, the
solvent was removed under reduced pressure, and the crude product was purified by column chromatography on silica gel with 3% MeOH:CH$_2$Cl$_2$ as the eluent to yield **112d** (179 mg, 82%) as a sticky oil. TLC $R_f = 0.75$ (CHCl$_3$:MeOH, 10:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.50 (s, 1H, CH), 8.33 (d, J = 8.4 Hz, 1H, NH), 8.22 (t, J = 1.5 Hz, 1H, Ar), 7.33 (dd, J = 11.5, 1.7 Hz, 1H, Ar), 7.01 (d, J = 2.8 Hz, 1H, Ar), 6.38 (s, 2H, CH$_2$), 6.23 (dd, J = 2.8, 0.5 Hz, 1H, Ar), 4.82 (td, J = 8.2, 4.9 Hz, 1H, CH), 4.44 (t, J = 7.0 Hz, 2H, CH$_2$), 4.25 (qd, J = 7.1, 2.5 Hz, 2H, CH$_2$), 4.12 (q, J = 7.2 Hz, 2H, CH$_2$), 3.15 (s, 3H, CH$_3$), 3.06 (d, J = 0.6 Hz, 3H, CH$_3$), 2.73 (t, J = 7.8 Hz, 2H, CH$_2$), 2.55 – 2.41 (m, 2H, CH$_2$), 2.40 – 2.09 (m, 2H, CH$_2$), 1.98 – 1.87 (m, 2H, CH$_2$), 1.71 – 1.63 (m, 2H, CH$_2$), 1.32 (t, J = 7.1 Hz, 3H, CH$_3$), 1.28 – 1.23 (m, 3H, CH$_3$), 1.18 (s, 9H, CH$_3$). The intermediate **112d** was used for the next reaction without further characterization.

Diethyl (5-(5-((dimethylamino)methylene)amino)-4-oxo-3-((pivaloyloxy)methyl)-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)-3-fluoropicolinoyl)-L-glutamate (**113d**).

Compound **113d** was prepared using above procedure from **92d** (0.4 g, 1.25 mmol), NaH (50 mg, 1.25 mmol), and **110d** (0.79 g, 1.5 mmol) to give **113d** in 80% (715 mg) yield as a yellow oil. TLC $R_f = 0.77$ (CHCl$_3$:MeOH, 10:1) $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.51 (s, 1H, CH), 8.35 (d, J = 8.2 Hz, 1H, NH), 8.25 (t, J = 1.6 Hz, 1H, NH), 7.33 (dd, J = 11.3, 1.8 Hz, 1H, Ar), 7.01 (d, J = 2.5 Hz, 1H, Ar), 6.39 (s, 2H, CH$_2$), 6.23 (dd, J = 2.7, 0.7 Hz, 1H, Ar), 4.85 (td, J = 7.9, 5.1 Hz, 1H, CH), 4.41 (t, J = 7.0 Hz, 2H, CH$_2$), 4.25 (q, J = 6.7 Hz, 2H, CH$_2$), 4.12 (q, J = 7.3 Hz, 2H, CH$_2$), 3.15 (s, 3H, CH$_3$), 3.06 (s, 3H, CH$_3$), 2.76 (t, J = 7.4 Hz, 2H, CH$_2$), 2.54 (m, 2H, CH$_2$), 2.41 - 2.28 (m, 2H, CH$_2$), 1.95-1.78 (m, 2H, CH$_2$), 1.72 – 1.62 (m, 4H, CH$_2$), 1.32 (t, J = 7.1 Hz, 3H, CH$_3$), 1.28 – 1.23 (m, 3H, CH$_3$), 1.18 (s, 9H, CH$_3$). The intermediate **113d** was used for the next reaction without further characterization.
(5-(4-(2-amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)-3-fluoropicolinoyl)-L-glutamic acid (23c).

To a 25 mL round-bottomed flask was added 112d (0.24 g, 0.34 mmol), 1N NaOH (4.12 mL, 4.12 mmol) and MeOH (5 mL), stirred overnight. MeOH was evaporated and reaction was acidified to pH 5 using 1N HCl to afford 23c (133 mg, 82%) as a white precipitate. mp 226.8°C; 1H NMR (400 MHz, DMSO-d6) δ 8.74 (d, J = 8.0 Hz, 1H, NH), 8.35 (s, 1H, Ar), 7.71 (dd, J = 12.0, 1.6 Hz, 1H, Ar), 7.21 (d, J = 2.8 Hz, 1H, Ar), 5.89 (d, J = 2.8 Hz, 1H, Ar), 5.78 (s, 2H, 2-NH2), 4.41 (td, J = 8.5, 5.1 Hz, 1H, CH), 4.26 (t, J = 6.8 Hz, 2H, CH2), 2.70 (t, J = 7.6 Hz, 2H, CH2), 2.31 (t, J = 7.4 Hz, 2H, CH2), 2.03 (m, 2H, CH2), 1.74 (p, J = 6.9 Hz, 2H, CH2), 1.52 (p, J = 7.6 Hz, 2H, CH2). Mass calculated for C21H23FN6O6 [M + H]+, 475.2; Found 475.6. HPLC analysis: retention time, 7.81 min; peak area, 97.08 %; eluent A, H2O with 0.1% TFA: eluent B, ACN with 0.1% TFA; gradient elution (95% H2O to 0 % H2O) over 17 min with flow rate of 0.3 mL/min and detection at 245 nm; column temperature, rt.

(5-(5-(2-amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)-3-fluoropicolinoyl)-L-glutamic acid (24c).

To a 25 mL round-bottomed flask was added 113d (0.48 g, 0.67 mmol), 1N NaOH (8.07 mL, 8.07 mmol) and MeOH (5 mL), stirred overnight. MeOH was evaporated and reaction was acidified to pH 5 using 1N HCl to afford 24c (262 mg, 80%) as a white precipitate. mp 190.3°C; 1H NMR (400 MHz, DMSO-d6) δ 8.75 (d, J = 7.9 Hz, 1H, NH), 8.35 (s, 1H, Ar), 7.73 (dd, J = 11.9, 1.6 Hz, 1H, Ar), 7.18 (d, J = 2.8 Hz, 1H, Ar), 5.88 (d, J = 2.8 Hz, 1H, Ar), 5.86 (s, 2H, 2-NH2), 4.41 (td, J = 8.5, 5.1 Hz, 1H, CH), 4.21 (t, J = 7.0 Hz, 2H, CH2), 2.67 (t, J = 7.7 Hz, 2H, CH2), 2.31 (t, J = 7.4 Hz, 2H, CH2), 2.17 – 1.91 (m, 2H, CH2), 1.75 (p, J = 7.1 Hz, 2H, CH2), 1.61 (p, J = 7.7 Hz, 2H, CH2), 1.29 – 1.16 (m, 2H, CH2). Mass calculated for C22H25FN6O6 [M + H]+, 489.1; Found 489.1. HPLC analysis: retention time, 1.15 min; peak area, 98.43 %; eluent A, H2O with 0.1% TFA: eluent B, ACN with 0.1% TFA; gradient elution
(95% H$_2$O to 0 % H$_2$O) over 17 min with flow rate of 0.3 mL/min and detection at 245 nm; column temperature, rt.

Methyl 5-(5-oxohexyl)picolinate (116d).

To a solution of methyl 4-bromopicolinate (118d) (1.4 g, 5 mmol, 1 eq) in DMF (20 mL) was added hex-5-en-2-one 117d (0.52 mL, 5 mmol, 1 eq), LiCl (212 mg, 5 mmol, 1 eq), LiOAc (825 mg, 12.5 mmol, 2.5 eq), Bu$_4$NCl (1.41 g, 5 mmol, 1 eq), Pd(OAc)$_2$ (68 mg, 0.3 mmol, 0.06 eq) and the mixture was stirred at 70 °C for 4 hours. TLC showed the disappearance of the starting material and formation of one major polar spot (hexane/EtOAc). To the reaction mixture cooled to room temperature was added ethyl acetate. The resulting solution was extracted with H$_2$O and dried over Na$_2$SO$_4$. After evaporation of solvent, the residue was loaded on a silica gel column and flash-chromatographed with hexane followed by gradual increase in polarity to 100% EtOAc. The desired fractions were pooled, evaporated and the residue was dried in vacuo using P$_2$O$_5$ to afford mixture of 119d (720 mg, 60%) as colorless syrup. TLC $R_f$ = 0.42 (hexane/EtOAc 3:1) The crude $^1$H NMR of the intermediate 119d is mentioned in the chemical discussion section. The mixture was taken directly to reduction step.

To 10% palladium on activated carbon (1:1 wt equiv) in a Parr flask, ethanol was added to quench. Methanolic solutions of 119d (0.72 g, 4.3 mmol) were added, and hydrogenation was carried out at 55 psi of H$_2$ for 5 h. The reaction mixture was filtered through Celite®, washed with MeOH, passed through a short silica gel column (3 cm × 5 cm), and concentrated under reduced pressure to give methyl 5-(5-oxohexyl)picolinate (116d) (0.69 g, quant. yield) as clear oil. $R_f$ = 0.39 (hexane/EtOAc, 3:1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.58 (dd, J = 2.3, 0.7 Hz, 1H, Ar), 8.08 (dd, J = 7.9, 0.8 Hz, 1H, Ar), 7.67 (dd, J = 8.0, 2.3 Hz, 1H, Ar), 4.02 (s, 3H, OCH$_3$), 2.73 (t, J = 7.2 Hz, 2H, CH$_2$), 2.53 – 2.46 (m, 2H, CH$_2$), 2.15 (s, 3H, CH$_3$), 1.69 – 1.62 (m, 4H, CH$_2$). The intermediate 116d was used for the next reaction without further characterization.
Methyl 5-(5-(2-(2-amino-6-oxo-1,6-dihydropyrimidin-4-yl)hydrazoneylidene)hexyl)pyridylcarbonate (121d).

A mixture of the ketone 119d (591 mg, 2.52 mmol, 1 eq) and 2-Amino-6-hydrazinopyrimidin-4(3H)-one 115d (356 mg, 2.52 mmol, 1 eq) in 2-methoxyethanol (20 mL) was refluxed for 14 h, then cooled to room temperature and filtered. The filtrate was concentrated to which 1:1 ether/hexanes were added, and the resulting brownish powder was collected by filtration to afford 121d (770 mg, 89%) as a light brown powder. TLC Rf = 0.2 (CHCl3/MeOH, 5:1); mp 183 °C. 1H NMR (400 MHz, DMSO-d6) δ 9.95 (s, 1H, NH), 8.72 (s, 1H, NH), 8.58 (d, J = 2.3 Hz, 1H, Ar), 7.99 (d, J = 8.0 Hz, 1H, Ar), 7.83 (dd, J = 8.0, 2.2 Hz, 1H, Ar), 5.08 (s, 1H, Ar), 4.44 – 4.38 (m, 2H, CH2), 3.84 (s, 3H, OCH3), 3.68 – 3.64 (m, 2H, CH2), 3.30 (s, 3H, CH3), 2.72 (t, J = 7.5 Hz, 2H, CH2), 1.68 – 1.58 (m, 2H, CH2). Mass calculated for C17H22N6O3 [M - H]-, 357.14; Found 357.2.


To a suspension of an E/Z mixture of 3-aminobut-2-enenitrile 122d (4 g, 48.72 mmol) in MeOH (60 mL) was added diethyl aminomalonate hydrochloride 123d (10.72 g, 50.7 mmol). The resulting mixture was stirred at room temperature for 5 h. TLC showed the disappearance of the starting materials and the formation of one major spot at TLC Rf = 0.26 (EtOAc/ hexane, 1:2). The reaction solvent was diluted with ethyl acetate (50 mL), washed with brine (30 mL × 2), and dried over MgSO4. To the organic solvent 15 g of silica gel was added, and the mixture was evaporated to dryness under reduced pressure. This silica gel plug was loaded on a dry silica gel column (2 cm × 15 cm) and flash-chromatographed initially with n-hexane and then sequentially with 5% ethyl acetate in n-hexane, 10% ethyl acetate in n-hexane, and 15% ethyl acetate in n-hexane. Fractions containing the desired product (TLC) were pooled and evaporated to afford 9.38 g (80%) of 124d as an off-white solid: mp 51-53 °C; 1H NMR (400 MHz, DMSO-d6) δ E isomer 4.96 (d, 1 H, J = 7.8 Hz, CH), 4.18–4.24 (m, 4 H, CH2), 3.94 (s,
1 H, CH), Z isomer 7.51–7.56 (d, 2 H, J = 7.8 Hz, CH), 5.32 (s, 1 H), 2.05 (s, 6 H, CH₃), 1.18–1.23 (t, 3 H, J = 6.9 Hz, CH₃).

Ethyl 3-Amino-5-methyl-1H-pyrrole-2-carboxylate (125d).

A solution of NaOEt in EtOH (0.5 M, 150 mL) was added slowly to a stirred solution of 124d (6 g, 25 mmol) in 120 mL of EtOH. The reaction mixture was stirred for 6 h at 60 °C and cooled to room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with 10% EtOAc/Hexane as the eluent to yield 125d (3.17 g, 75%) as an off-white solid: mp 89-92 °C TLC Rf = 0.36 (EtOAc/Hexane, 1:1); ¹H NMR (400 MHz, DMSO-d₆) δ 10.21 (s, 1 H, NH), 5.26 (s, 1 H, Ar), 4.91 (s, 2 H, 2-NH₂), 4.12 (q, 2 H, J = 6.4 Hz, CH₂), 4.12 (q, 2 H, J = 6.4 Hz, CH₂), 2.03 (s, 3H, CH₃), 1.24 (t, 3 H, J = 6.4 Hz, CH₃).

5-(4-(2-Amino-6-methyl-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinic acid (128d).

To a solution of ethyl 3-amino-5-methyl-1H-pyrrole-2-carboxylate 125d (0.75 g, 4.5 mmol) in dry DMF (10 mL) was added slowly NaH (0.21 g, 5.4 mmol) under nitrogen at room temperature. The resulting mixture was stirred for about 15 min when there was no more gas produced, and then methyl 5-(4-iodobutyl)picolinate (1.42 g, 4.5 mmol) in DMF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 h, and 20 mL of water was added carefully to quench the reaction. The sample was extracted with EtOAc (25 mL × 4), and the organic phases were combined and dried (Na₂SO₄). Evaporation of the solvent offered a gummy residue, which was used for the next step without purification.

The gummy residue (~1.72 mmol) was dissolved in MeOH (20 mL), and 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea (0.36 g, 1.72 mmol) was added followed by AcOH (0.5 mL, 8.62mmol). The mixture was stirred at room temperature overnight and
became a thick paste. NaOMe in MeOH (25%) (2.6 mL, 12.07 mmol) was added, and stirring was continued at room temperature overnight. The mixture was neutralized with AcOH and the methanol was removed under reduced pressure. To the residue was added water (20 mL), and the pH value was adjusted to 10–11 by adding NH₃ · H₂O. The solid was collected by filtration and washed well with water. The resulting solid was added to 1 N NaOH (2 mL), and the mixture was heated at 55 °C for 3 h. The mixture was cooled and neutralized with acetic acid to give a precipitate. The solid was collected by filtration and chromatographed and eluted with 5% MeOH in CHCl₃ (containing 0.5% NH₃ · H₂O). Fractions containing the desired compound (TLC) were pooled and evaporated to afford 462 mg of 128d (25% for three steps) as an off white solid: mp >250 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 10.95 (s, 1H, NH), 8.40 (d, J = 7.6 Hz, 1H, Ar), 8.28 (d, J = 2.2 Hz, 1H, Ar), 7.88 (m, 2H, Ar), 5.74 – 5.66 (m, 3H, CH₃), 4.25 (qd, J = 7.1, 2.5 Hz, 2H, CH₂), 2.70 (d, J = 7.6 Hz, 2H, CH₂), 1.66 (d, J = 7.5 Hz, 2H, CH₂), 1.58 (d, J = 8.2 Hz, 2H, CH₂). Mass calculated for C₁₇H₁₉N₅O₃ [M + H]⁺, 342.15; Found 341.9.

Diethyl (5-(4-(2-amino-6-methyl-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinoyl)-l-glutamate (129d).

Compound 129d (122 mg, 64%) was prepared as a dark brown oil using similar glutamate coupling conditions as 29d, from 128d (100 mg, 0.5 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (104 mg, 0.6 mmol), 4-methylmorpholine (0.07 mL, 0.6 mmol) and diethyl L-glutamate HCl (109 mg, 0.7 mmol). TLC Rᵣ = 0.53 (CHCl₃:MeOH, 10:1). ¹H NMR (400 MHz, DMSO-d₆) δ 8.89 (d, J = 7.6 Hz, 1H, NH), 8.50 (d, J = 2.2 Hz, 1H, Ar), 7.93 (d, J = 7.8 Hz, 1H, Ar), 7.80 (dd, J = 8.2, 2.1 Hz, 1H, Ar), 5.71 (s, 1H, Ar), 5.69 (2, 2H, 2-NH₂), 4.53(m, 1H, CH), 4.25 (qd, J = 7.1, 2.5 Hz, 2H, CH₂), 4.12 (q, J = 7.2 Hz, 2H, CH₂), 4.05 (q, J = 7.0 Hz, 2H, CH₂), 2.70 (d, J = 7.6 Hz, 2H, CH₂), 2.36 – 2.29 (m, 2H, CH₂), 2.21 (s, 3H, CH₃), 2.06 (dd, J = 13.9, 7.4 Hz, 2H, CH₂), 1.66 (d, J = 7.5 Hz, 2H, CH₂), 1.58 (d, J = 8.2 Hz, 2H). 1.35
(t, J = 7.4 Hz, 3H, CH₃), 1.25 (m, 3H, CH₃). Mass calculated for C₂₆H₃₄N₆O₆ [M + H]⁺, 527.26; Found 527.2. The intermediate 129d was used for the next reaction without further characterization.

(5-(4-(2-Amino-6-methyl-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamic acid (26c).

To a 25 mL round-bottomed flask was added 129d (122 mg, 0.23 mmol), 1N NaOH (0.7 mL, 0.7 mmol) and MeOH (1 mL), stirred overnight. MeOH was evaporated and reaction was acidified to pH 5 using 1N HCl to afford 26c (80 mg, 73%) as a white precipitate. mp 223.3 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.77 (d, J = 7.6 Hz, 1H, NH), 8.50 (d, J = 2.2 Hz, 1H, Ar), 7.93 (d, J = 7.8 Hz, 1H, Ar), 7.80 (dd, J = 8.2, 2.1 Hz, 1H, Ar), 5.74 – 5.66 (m, 3H, 1Ar and 2-NH₂), 4.36 (d, J = 7.4 Hz, 1H, CH), 4.24 (t, J = 7.0 Hz, 2H, CH₂), 2.70 (d, J = 7.6 Hz, 2H, CH₂), 2.36 – 2.29 (m, 2H, CH₂), 2.23 (s, 3H, CH₃), 2.06 (t, J = 7.4 Hz, 2H, CH₂), 1.66 (d, J = 7.5 Hz, 2H, CH₂), 1.58 (d, J = 8.2 Hz, 2H, CH₂). Mass calculated for C₂₂H₂₆N₆O₆ [M + H]⁺, 471.2; Found 471.6. HPLC analysis: retention time, 8.13 min; peak area, 95.24%; eluent A, H₂O with 0.1% TFA; eluent B, ACN with 0.1% TFA; gradient elution (95% H₂O to 0% H₂O) over 17 min with flow rate of 0.3 mL/min and detection at 245 nm; column temperature, rt.

5-Bromo-2,4-di-tert-butoxypyrimidine (131d).

5-Bromo-2,4-dichloropyrimidine (2.28 g, 10.0 mmol) is dissolved in dry THF (50 mL). Then a solution of sodium tert-butoxide (2.4 g, 25.0 mmol) in THF (100 mL) is added dropwise at rt and the reaction mixture stirred for 5 h. The reaction is quenched with water (50 mL) and extracted with EtOAc (3 x 50 mL). Purification by flash chromatography yielded the desired product 131d as colorless solid (2.63 g, 87%). mp 63 °C. ¹H NMR (400 MHz, Chloroform-d) δ 8.25 (s, 1H, Ar), 1.66 (s, 9H, CH₃), 1.61 (s, 9H, CH₃).

General procedure for the preparation of benzylic phosphates (132d-136d):
To a flask charged with the requisite benzyl alcohols (20 mmol), triethylamine (4.18 mL, 30.0 mmol), DMAP (244 mg, 2 mmol) and THF (5 mL) was added neat diethyl chlorophosphate (2.88 mL, 20 mmol) over a 30 min period at room temperature. An exotherm was observed, generally raising the internal temperature to 30-35 °C. The resultant white, heterogeneous mixture was stirred for 5 h before it was poured into a mixture of 2 M KHSO₄ (20 mL) and water (30 mL). The separated organic phase was then washed with satd. aq. NaHCO₃ (30 mL) and brine (30 mL), before it was dried over MgSO₄, filtered and concentrated to leave a crude oil. The crude oil was purified by chromatography using appropriate mixtures of EtOAc/hexane (determined by TLC analysis) to elute any unreacted benzyl alcohol followed by the desired benzylic phosphate.

3,4-Dimethoxybenzyl diethyl phosphate (132d)

Following the above general procedure, 132d (3.7 g) was obtained as a pale yellow oil (60%) from 3,4-dimethoxybenzyl alcohol (3.4 g, 20 mmol). ¹H NMR (400 MHz, Chloroform-d) δ ¹H NMR (400 MHz, Chloroform-d) δ 6.92 (d, J = 1.9 Hz, 1H, Ar), 6.90 (d, J = 8.1 Hz, 1H, Ar), 6.88 – 6.83 (m, 1H, Ar), 4.98 (d, J = 8.2 Hz, 2H, CH₂), 3.90 (s, 3H, OCH₃), 3.92 (s, 3H, OCH₃), 4.18 – 4.12 (m, 4H, CH₂), 1.34 (td, J = 7.1, 1.0 Hz, 6H, CH₃).

4-Methylthiobenzyl diethyl phosphate (133d)

Following the above general procedure, 133d (2.35 g) was obtained as a pale yellow oil (40%) from 4-methylthiobenzyl alcohol (3.1 g, 20 mmol). ¹H NMR (400 MHz, Chloroform-d) δ 7.24 – 7.20 (m, 2H, Ar), 7.17 – 7.11 (m, 2H, Ar), 5.21 (d, J = 8.2 Hz, 2H, CH₂), 4.16 – 4.08 (m, 4H, CH₂), 2.53 (s, 3H, CH₃), 1.35 (t, J = 7.1 Hz, 6H, CH₃).

3,4-Difluorobenzyl diethyl phosphate (134d).

Following the above general procedure, 134d (4.01 g) was obtained as a pale yellow oil (70%) from 3,4-difluorobenzyl alcohol (2.95 g, 20 mmol): ¹H NMR (400 MHz, Chloroform-d) δ 7.10 (dt, J = 10.3, 8.3 Hz, 1H, Ar), 7.01 (ddd, J = 11.4, 7.6, 2.2 Hz, 1H, Ar), 6.88 (ddt, J = 8.2, 3.9,
1.7 Hz, 1H, Ar), 4.98 (d, J = 8.2 Hz, 2H, CH₂), 4.18 – 4.11 (m, 4H, CH₂), 1.36 (t, J = 7.1 Hz, 6H, CH₃).

3,4,5-Trifluorobenzyl diethyl phosphate (135d).

Following the above general procedure, 135d (4.08 g) was obtained as a pale yellow oil (75%) from 3,4,5-trifluorobenzyl alcohol (2.95 g): ¹H NMR (400 MHz, Chloroform-d) δ 7.05 (t, J = 7.9 Hz, 2H, Ar), 5.00 (d, J = 8.2 Hz, 2H, CH₂), 4.19 – 4.09 (m, 4H, CH₂), 1.36 (t, J = 7.1 Hz, 6H, CH₃).

4-(trifluoromethoxy)benzyl diethyl phosphate (136d).

Following the above general procedure, 136d (4.4 g) was obtained as a pale yellow oil (68%) from 4-(trifluoromethoxy)benzyl alcohol (3.8 g, 20 mmol): ¹H NMR (400 MHz, Chloroform-d) δ 6.88 (dd, J = 8.5, 6.5 Hz, 2H, Ar), 6.76 (dd, J = 8.5, 6.5 Hz, 2H, Ar), 5.01 (d, J = 8.2 Hz, 2H, CH₂), 4.16 (m, 4H, CH₂), 1.36 (t, J = 7.3 Hz, 6H, CH₃).

**General procedure for the preparation of 137d-141d:**

In a flame-dried microwave vial (20 mL) under nitrogen, 131d (1 eq) is dissolved in DME (3 mL) and cooled to 0 °C. iPrMgCl (1.3 M in THF, 1.1 eq) is added dropwise via syringe. After 20 min at 0 °C, CuBr (0.1 eq) and P(OEt)₃ (0.2 eq) are added. This mixture is added over 30 min via cannula to a mixture of benzyl phosphates (1.1 eq) and TBAI (0.1 eq) at 60 °C. The reaction mixture is heated at 60 °C for 1 h. The reaction mixture is quenched with saturated NH₄Cl solution, extracted with ether, and dried (Na₂SO₄), and the crude product was purified by flash chromatography (30% EtOAc/Hexane).

2,4-Di-tert-butoxy-5-(3,4-dimethoxybenzyl)pyrimidine (137d).

Following the above general procedure, 137d was obtained as a clear oil (1.1 g, 54 %) from 131d (1.2 g, 6.25 mmol): ¹H NMR (400 MHz, Chloroform-d) δ 7.99 (d, J = 1.7 Hz, 1H, Ar), 6.92 (d, J = 1.9 Hz, 1H, Ar), 6.90 (d, J = 8.1 Hz, 1H, Ar), 6.88 – 6.83 (m, 1H, Ar), 3.92 (s, 3H, OCH₃), 3.90 (s, 3H, OCH₃), 3.68 (s, 2H, CH₂), 1.61 (s, 9H, CH₃), 1.55 (s, 9H, CH₃).
2,4-Di-tert-butoxy-5-(4-(methylthio)benzyl)pyrimidine (138d).

Following the above general procedure, 138d was obtained as a clear oil (770 mg, 40 %) from 131d (1.1 g, 5.73 mmol): \( ^1 \text{H NMR (400 MHz, Chloroform-}d\text{)} \) \( \delta \) 7.99 (s, 1H, Ar), 7.26 – 7.17 (m, 2H, Ar), 7.17 – 7.08 (m, 2H, Ar), 3.68 (s, 2H, CH\(_2\)), 2.48 (s, 3H, SCH\(_3\)), 1.61 (s, 9H, CH\(_3\)), 1.55 (s, 9H, CH\(_3\)).

2,4-Di-tert-butoxy-5-(3,4-difluorobenzyl)pyrimidine (139d).

Following the above general procedure, 139d was obtained as a clear oil (1.03 g, 43 %) from 131d (1.4 g, 7.3 mmol): \( ^1 \text{H NMR (400 MHz, Chloroform-}d\text{)} \) \( \delta \) 7.99 (s, 1H, Ar), 7.05 (dt, \( J = 10.3 \), 8.3 Hz, 1H, Ar), 6.99 (ddd, \( J = 11.4 \), 7.6, 2.2 Hz, 1H, Ar), 6.89 (ddt, \( J = 8.2 \), 3.9, 1.7 Hz, 1H, Ar), 3.66 (s, 2H, CH\(_2\)), 1.61 (s, 9H, CH\(_3\)), 1.54 (s, 9H, CH\(_3\)).

2,4-Di-tert-butoxy-5-(3,4,5-trifluorobenzyl)pyrimidine (140d).

Following the above general procedure, 140d was obtained as a clear oil (1.77 g, 57 %) from 131d (1.8 g, 9.37 mmol): \( ^1 \text{H NMR (400 MHz, Chloroform-}d\text{)} \) \( \delta \) 8.02 (s, 1H, Ar), 6.80 (dd, \( J = 8.5 \), 6.5 Hz, 2H, Ar), 3.65 (s, 2H, CH\(_2\)), 1.63 (s, 9H, CH\(_3\)), 1.56 (s, 9H, CH\(_3\)).

2,4-Di-tert-butoxy-5-(4-(trifluoromethoxy)benzyl)pyrimidine (141d).

Following the above general procedure, 141d was obtained as a clear oil (1.48 g, 60 %) from 131d (1.43 g, 7.44 mmol): \( ^1 \text{H NMR (400 MHz, Chloroform-}d\text{)} \) \( \delta \) 8.02 (s, 1H, Ar), 6.88 (dd, \( J = 8.5 \), 6.5 Hz, 2H, Ar), 6.76 (dd, \( J = 8.5 \), 6.5 Hz, 2H, Ar), 3.65 (s, 2H, CH\(_2\)), 1.61 (s, 9H, CH\(_3\)), 1.56 (s, 9H, CH\(_3\)).

**General procedure for the preparation of 5-substituted-benzyl pyrimidine-2,4-diamine (28c-32c).**

2,4-Di-tert-butoxy-5-substituted-benzyl pyrimidines (137d-141d) were dissolved in MeOH (5 mL) then conc. HCl (2 mL) was added dropwise and immediately a precipitate was formed. The reaction mixture was stirred 15 min at rt, then the precipitate was filtered off and
washed with cold MeOH and dried in vacuo to yield the desired products 5-(substituted-benzyl)pyrimidine-2,4-(1H,3H)-dione as colorless solids in 80-90% yield. The compounds were used for the next step without further purification.

To a stirred mixture of POCl₃ (15 mL) and 5-(substituted-benzyl)pyrimidine-2,4(1H,3H)-diones in a 25 mL round-bottomed flask with reflux condenser 2 drops of DMF were added then the reaction mixture was refluxed for 1 h at 110 °C. Afterwards the reaction mixture was poured on ice, extracted with diethyl ether, and the organic layer dried over Na₂SO₄. Purification by flash chromatography yielded 142d-146d as colorless solids.

Intermediates 142d-146d were suspended in a solution of NH₃ in MeOH (10 mL, 7 N) in a 10-20 mL microwave vial at 150 °C for 12 h (19-20 bar pressure), then the solvent is removed in vacuo. Crude was purified by column chromatography to yield proposed compounds (28c-32c) in 30-40% yield over three steps.

5-(3,4-Dimethoxybenzyl)pyrimidine-2,4-diamine (28c).

Following the above general procedure, 28c was obtained as a cream white solid (30 %): ¹H NMR (400 MHz, DMSO-d₆) δ 7.51 (s, 1H, Ar), 6.91 (d, J = 1.9 Hz, 1H, Ar), 6.89 (d, J = 8.1 Hz, 1H, Ar), 6.85 (m, 1H, Ar), 6.12 (s, 2H, 4-NH₂), 5.75 (s, 2H, 2-NH₂), 3.92 (s, 3H, OCH₃), 3.90 (s, 3H, OCH₃), 3.62 (s, 2H, CH₂). mp 231-233 °C (lit. 369 233 °C). Anal. Calcd for C₁₃H₁₆N₄O₂: C, 59.98; H, 6.19; N, 21.52. Found: C, 59.75; H, 6.22; N, 21.37.

5-(4-(Methylthio)benzyl)pyrimidine-2,4-diamine (29c).

Following the above general procedure, 29c was obtained as a white solid (37 %): ¹H NMR (400 MHz, DMSO-d₆) δ 7.48 (s, 1H, Ar), 7.17 (s, 4H, Ar), 6.10 (s, 2H, 4-NH₂), 5.73 (s, 2H, 2-NH₂), 3.56 (s, 2H, CH₂), 2.44 (s, 3H, SCH₃). mp 211-214 °C. Anal. Calcd for C₁₂H₁₄N₄S · 0.2715 CH₃OH: C, 57.79; H, 5.96; N, 21.97; S, 12.57. Found: C, 58.08; H, 5.69; N, 21.58; S, 12.62.
5-(3,4-Difluorobenzyl)pyrimidine-2,4-diamine (30c).

Following the above general procedure, 30c was obtained as a white solid (40 %): $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.55 (s, 1H, Ar), 7.38 – 7.22 (m, 2H, Ar), 7.09 – 7.00 (m, 1H, Ar), 6.16 (s, 2H, 4-NH$_2$), 5.76 (s, 2H, 2-NH$_2$), 3.60 (s, 2H, CH$_2$). mp 189 °C. Anal. Calcd for C$_{11}$H$_{10}$F$_2$N$_4$: C, 55.92; H, 4.26; N, 23.72; F, 16.08. Found: C, 55.98; H, 4.41; N, 23.54; F, 15.95.

5-(3,4,5-Trifluorobenzyl)pyrimidine-2,4-diamine (31c).

Following the above general procedure, 31c was obtained as a buff color solid(36 %): $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.53 (s, 1H, Ar), 7.40 – 7.33 (t, J = 5.6 Hz, 2H, Ar), 6.13 (s, 2H, 4-NH$_2$), 5.74 (s, 2H, 2-NH$_2$), 3.62 (s, 2H, CH$_2$). mp 222-224 °C. Anal. Calcd for C$_{11}$H$_9$F$_3$N$_4$: C, 51.97; H, 3.57; N, 22.04, F, 22.42. Found: C, 51.63; H, 3.45; N, 21.85; F, 22.08.

5-(4-(Trifluoromethoxy)benzyl)pyrimidine-2,4-diamine (32c).

Following the above general procedure, 32c was obtained as a white solid (34 %): $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.52 (s, 1H, Ar), 7.00 (dd, J = 8.3, 6.1 Hz, 2H, Ar), 6.82 (dd, J = 8.3, 6.4 Hz, 2H, Ar), 6.11 (s, 2H, 4-NH$_2$), 5.75 (s, 2H, 2-NH$_2$), 3.65 (s, 2H, CH$_2$). mp 264.7 °C. Anal. Calcd for C$_{12}$H$_{11}$F$_3$N$_4$O: C, 50.70; H, 3.90; N, 19.71; F, 20.05. Found: C, 50.93; H, 4.09; N, 19.50 F, 19.78.
VI. SUMMARY

This dissertation describes the design and synthesis of selective pjDHFR inhibitors, single agents with combination chemotherapy and multiple RTK inhibitory potential 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. \( N-(5\text{-Methoxynaphthalen-2-yl})-N\text{-methylquinazolin-4-amine (1c)} \).
2. \( 2\text{-Chloro-}N\text{-methyl-}N-(4\text{-}(methylthio)phenyl)\text{quinazolin-4-amine (2c).} \)
3. \( 2\text{-Chloro-}N\text{-}(4\text{-methoxyphenyl})\text{-N-methylpyrido[3,2-d]pyrimidin-4-amine (3c).} \)
4. \( 4\text{-}(6\text{-Methoxy-3,4-dihydroquinolin-1(2H)-yl})\text{pyrido[3,2-}d\text{]pyrimidin-4-amine (4c).} \)
5. \( 2\text{-Chloro-}N\text{-methyl-N-}4\text{-}(methylthio)phenyl)\text{pyrido[3,2-}d\text{]pyrimidin-4-amine (5c).} \)
6. \( 2\text{-Chloro-N-(5} \text{-methoxynaphthalen-2-yl} \text{-N-methylpyrido[3,2-}d\text{]pyrimidin-4-amine (6c).} \)
7. \( N\text{-}(4\text{-Methoxyphenyl})\text{-N-methylpyrido[3,2-}d\text{]pyrimidin-4-amine (7c).} \)
8. \( 4\text{-}(6\text{-Methoxy-3,4-dihydroquinolin-1(2H)-yl})\text{pyrido[3,2-}d\text{]pyrimidin-4-amine (8c).} \)
9. \( N\text{-Methyl-N-}4\text{-}(methylthio)phenyl)\text{pyrido[3,2-}d\text{]pyrimidin-4-amine (9c).} \)
10. \( N\text{-}(5\text{-Methoxynaphthalen-2-yl})\text{-N-methylpyrido[3,2-}d\text{]pyrimidin-4-amine (10c).} \)
11. \( 5\text{-Phenoxy-}9H\text{-pyrimido[4,5-b]indole-2,4-diamine (11c).} \)
12. \( 5\text{-}(p\text{-Tolyloxy)-}9H\text{-pyrimido[4,5-b]indole-2,4-diamine (12c).} \)
13. \( (5\text{-}(2\text{-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-}d\text{]pyrimidin-5-yl})\text{ethyl} \text{picolinoyl})-L\text{-glutamic acid (13c).} \)
14. \( (5\text{-}(3\text{-}(2\text{-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-}d\text{]pyrimidin-5-yl})\text{propyl} \text{picolinoyl})-L\text{-glutamic acid (14c).} \)
15. (5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamic acid (15c).

16. (5-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)picolinoyl)-L-glutamic acid (16c).

17. (6-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)nicotinoyl)-L-glutamic acid (17c).

18. (6-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)nicotinoyl)-L-glutamic acid (18c).

19. (6-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)nicotinoyl)-L-glutamic acid (19c).

20. 5-(3,4-Dimethoxybenzyl)pyrimidine-2,4-diamine (20c).

21. 5-(4-(Methylthio)benzyl)pyrimidine-2,4-diamine (21c).

22. 5-(3,4-Difluorobenzyl)pyrimidine-2,4-diamine (22c).

23. 5-(3,4,5-Trifluorobenzyl)pyrimidine-2,4-diamine (23c).

24. 5-(4-(Trifluoromethoxy)benzyl)pyrimidine-2,4-diamine (24c).

**Partially characterized final compounds:**

1. (5-(3-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)propyl)picolinoyl)-L-glutamic acid (20c).

2. (5-(4-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)picolinoyl)-L-glutamic acid (21c).

3. (5-(5-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)pentyl)picolinoyl)-L-glutamic acid (22c).

4. (5-(4-(2-Amino-4-oxo-3,4-dihydro-5H-pyrrolo[3,2-d]pyrimidin-5-yl)butyl)-3-fluoropicolinoyl)-L-glutamic acid (23c).
5. \((5-(5-(2\text{-}Amino\text{-}4-\text{oxo}\text{-}3,4\text{-}dihydro\text{-}5\text{H}\text{-}pyrrolo}[3,2\text{-}d]\text{pyrimidin}\text{-}5\text{yl})\text{penty}l)\text{-}3\text{-}\text{fluoropicolinoyl})\text{-}L\text{-}\text{glutamic acid (24c).}

6. \((5-(4-(2\text{-}Amino}\text{-}6\text{-}methyl\text{-}4\text{-}\text{oxo}\text{-}3,4\text{-}dihydro\text{-}5\text{H}\text{-}pyrrolo}[3,2\text{-}d]\text{pyrimidin}\text{-}5\text{yl})\text{butyl}\text{-}\text{picolinoyl})\text{-}L\text{-}\text{glutamic acid (26c).}

Among these compounds \(1\text{-}10c\) were evaluated as microtubule targeting agents binding at the colchicine site of tubulin. Compounds \(1c\) and \(7c\) were also tested for microtubule depolymerization and inhibition of tubulin assembly assays. Compounds \(3c\text{-}10c\) were tested against parental OVCAR-8 and P-gp overexpressing NCI/ADR-RES cell lines. RTK inhibitory assays for compounds \(1c\text{-}10c\) are underway.

During this study, a novel synthetic route was successfully optimized for the synthesis of target compounds \(11c\) and \(12c\). Ullmann coupling conditions were explored and successfully employed as a key step to obtain the target compounds.

Biological evaluation of proposed compounds \((13c\text{-}15c, 20c\text{-}22c\text{ and }26c)\) were carried out in engineered CHO cell lines that overexpressing RFC, FR\(\alpha\), FR\(\beta\), and PCFT. Testing of the other compounds \((16c\text{-}19c\text{ and }23c\text{-}24c)\) are underway. Majority of the proposed compounds showed improved selectivity compared to the lead compounds. Using traditional bioisosteric substitutions and possible conformation restriction (via intra-molecular H-bonding (pyridine nitrogen) or stereoelectronic effects (fluorine substitution)), we were able to successfully incorporate selectivity and improve potency. Evaluation of compounds \(28c\text{-}32c\) are underway for inhibition of pjDHFR and hDHFR.
VII. APPENDIX

Biological Evaluation

The tumor inhibitory assays, microtubule depolymerization assays were performed by Dr. Susan L. Mooberry (Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229). The colchicine binding assays and bovine brain tubulin polymerization assays were performed by 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). The biological evaluations of the were performed by Dr. Larry H. Matherly (Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute and the Cancer Biology Program and Department of Pharmacology, Wayne State University School of Medicine) against GARFTase, RFC-expressing PC43-10 cells, FRα-expressing RT16 cells, FRβ-expressing D4 cells and hPCFT-expressing R2/hPCFT4 cells.

Table 16. Antiproliferative and microtubule depolymerizing effects of quinazoline (1c) and pyrido[3,2-d]pyrimidine analog (7c).

<table>
<thead>
<tr>
<th>No.</th>
<th>IC₅₀ ± SD in MDA-MB-435 Cells (nM)</th>
<th>EC₅₀ (nM) for Microtubule Depolymerization in A-10 Cells</th>
<th>EC₅₀/IC₅₀ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>2.7 ± 0.5</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>7c</td>
<td>30.1 ± 3</td>
<td>272</td>
<td>9</td>
</tr>
<tr>
<td>CA-4</td>
<td>4.4 ± 0.3</td>
<td>9.8</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 17. Effects of pyrido[3,2-\textit{d}]pyrimidine analogs 3c-10c and CA-4 on tubulin assembly and colchicine binding. Growth inhibition of parental cell line (OVCAR-8) and Pgp overexpressing cell line (NCI/ADR-RES).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of tubulin assembly</th>
<th>Inhibition of colchicine binding</th>
<th>Compound activity in a Pgp overexpressing cell line (NCI/ADR-RES) and in a parental cell line (OVCAR-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (SD) µM</td>
<td>5 µM inhibitor</td>
<td>OVCAR-8 IC$_{50}$ (SD) nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 µM inhibitor</td>
<td>NCI/ADR-RES IC$_{50}$ (SD) nM</td>
</tr>
<tr>
<td>2c</td>
<td>0.5 (0.06)</td>
<td>96 (3)</td>
<td>6 (0.7)</td>
</tr>
<tr>
<td>3c</td>
<td>0.66 (0.07)</td>
<td>97 (0.06)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>4c</td>
<td>0.62 (0.1)</td>
<td>97 (3)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>5c</td>
<td>0.68 (0.02)</td>
<td>96 (0.5)</td>
<td>NT</td>
</tr>
<tr>
<td>6c</td>
<td>0.97 (0.008)</td>
<td>98 (0.1)</td>
<td>NT</td>
</tr>
<tr>
<td>7c</td>
<td>1.3 (0.04)</td>
<td>87 (0.6)</td>
<td>ND</td>
</tr>
<tr>
<td>8c</td>
<td>1.6 (0.09)</td>
<td>86 (1)</td>
<td>ND</td>
</tr>
<tr>
<td>9c</td>
<td>0.74 (0.1)</td>
<td>82 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>10c</td>
<td>0.8 (0.08)</td>
<td>92 (0.007)</td>
<td>NT</td>
</tr>
<tr>
<td>CA-4</td>
<td>0.54 (0.06)</td>
<td>99 (0.7)</td>
<td>9.0 (1)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>-</td>
<td>-</td>
<td>5.5 (2)</td>
</tr>
</tbody>
</table>

Paclitaxel - 3100 (800)
Table 18. IC₅₀ Values (nM) for 5-Substituted Pyrrolo[2,3-d]pyrimidine Classical Antifolates in RFC-, PCFT-, and FR-Expressing Cell Lines.

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFRα</th>
<th>hPCFT</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>RT16</td>
<td>R2/hPCFT4</td>
<td>hRFC/hFRα/FRβ/hPCFT</td>
</tr>
<tr>
<td>PMX</td>
<td>26.2 ± 5.5</td>
<td>42 ± 9</td>
<td>8.3 ± 2.7</td>
<td>68 ± 12</td>
</tr>
<tr>
<td>45c</td>
<td>68.8 ± 21.2</td>
<td>72.0 ± 27.1</td>
<td>329 ± 61</td>
<td>68.8 ± 21.2</td>
</tr>
<tr>
<td>46c</td>
<td>56.6 ± 5.8</td>
<td>8.6 ± 2.1</td>
<td>840 ± 90</td>
<td>56.6 ± 5.8</td>
</tr>
<tr>
<td>13c</td>
<td>89.5</td>
<td>336</td>
<td>10.3</td>
<td>497</td>
</tr>
<tr>
<td>14c</td>
<td>546</td>
<td>17.2</td>
<td>96.6</td>
<td>15.9</td>
</tr>
<tr>
<td>15c</td>
<td>55</td>
<td>2.56</td>
<td>65.96</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Table 19. IC₅₀ Values (nM) for 5-Substituted Pyrrolo[3,2-d]pyrimidine Classical Antifolates in RFC-, PCFT-, and FR-Expressing Cell Lines.

<table>
<thead>
<tr>
<th></th>
<th>hRFC</th>
<th>hFRα</th>
<th>hPCFT</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC43-10</td>
<td>RT16</td>
<td>R2/hPCFT4</td>
<td>hRFC/hFRα/FRβ/hPCFT</td>
</tr>
<tr>
<td>48c</td>
<td>43</td>
<td>50</td>
<td>25.2</td>
<td>6.7</td>
</tr>
<tr>
<td>49c</td>
<td>516</td>
<td>2.13</td>
<td>309</td>
<td>6.8</td>
</tr>
<tr>
<td>50c</td>
<td>&gt;1000</td>
<td>26.6</td>
<td>327</td>
<td>6.3</td>
</tr>
<tr>
<td>20c</td>
<td>&gt;1000</td>
<td>8</td>
<td>749</td>
<td>10.78</td>
</tr>
<tr>
<td>21c</td>
<td>&gt;1000</td>
<td>0.58</td>
<td>&gt;1000</td>
<td>0.75</td>
</tr>
<tr>
<td>22c</td>
<td>&gt;1000</td>
<td>9.47</td>
<td>&gt;1000</td>
<td>1.58</td>
</tr>
<tr>
<td>26c</td>
<td>&gt;1000</td>
<td>14.12</td>
<td>&gt;1000</td>
<td>227</td>
</tr>
</tbody>
</table>

Data Highlighted in yellow is raw and not yet final.
**Figure 82.** Protection studies for compound 14c (AGF315)

![Graph showing relative growth against inhibitor concentration for compound 14c (AGF315).]

**Figure 83.** Protection studies for compound 15c (AGF317)

![Graph showing relative growth against inhibitor concentration for compound 15c (AGF317).]
Figure 84. Protection studies for compound 20c (AGF363)

Figure 85. Protection studies for compound 22c (AGF370)
Methods

Effects of compounds on cellular microtubules: The effect of the compounds on interphase and mitotic microtubules were evaluated in A-10 cells using indirect immunofluorescence techniques, and the EC50 (concentration required to cause 50% loss of cellular microtubules) values were calculated from a minimum of three experiments.

Sulforhodamine B (SRB) assay: The cytotoxic and antiproliferative effects of the compounds against cancer cells was evaluated using SRB assay. MDA-MB-435 cells were used for determining IC50 values (concentration required to cause 50% inhibition of proliferation).

[3H] Colchicine displacement assay: The binding of [3H]colchicine to tubulin was performed by the DEAE-cellulose filter technique with a stack of two filters, as described in detail previously. In brief, reaction mixtures contained, in a 0.10 mL volume, 1.0 μM purified tubulin, 5.0 μM [3H]colchicine, potential inhibitor at 5.0 μM, 5% (v/v) dimethyl sulfoxide (the compound solvent), and other components previously found to stabilize the colchicine binding activity of tubulin for prolonged periods at 37°C. Incubation was at 37°C for 10 min, at which time samples were diluted with 2 mL of ice-cold water and poured over the DEAE-filters under mild suction, with several rinses of the reaction vessel and of the filtration chamber. The amount of radiolabel bound to the filters was determined by liquid scintillation counting, and samples containing test compounds were compared to reaction mixtures without compound. The percent inhibition relative to the control was determined for each compound in 2-4 independent experiments.
OVCAR-8, NCI/ADR-RES and HeLa cells

The OVCAR-8 and the Pgp overexpressing NCI/ADR-RES cell lines were generously provided by the Drug Screening group of the Developmental Therapeutics Program, NCI. The wild-type and β-III overexpressing HeLa cells were generous gifts, respectively, of Dr. Richard F. Ludueña and Dr. Susan L. Mooberry. The OVCAR-8 and NCI/ADR-RES cells were grown in RPMI 1640 medium with 5% fetal bovine serum at 37 °C in a 5% CO2 atmosphere for 96 h in the presence of varying compound concentrations. The HeLa cells were grown in MEM supplemented with Earle’s salts, nonessential amino acids, 2 mM L-glutamine, and 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere for 96 h in the presence of varying compound concentrations. In all cultures, the DMSO concentration was 0.5%. Protein was the parameter measured by the sulforhodamine B technique,8 and the IC50 was defined as the compound concentration causing a 50% reduction in the increase in cell protein as compared with cultures without compound addition.

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 α-MEM 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, 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.**[^3H]Folic acid binding was used to assess levels of surface FRs. Briefly, cells (e.g., RT16 or D4; ~1.6 × 10⁶) were rinsed twice with Dulbecco’s phosphatebuffered saline (DPBS) followed by two washes in acidic buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) to remove FR-bound folates. Cells were washed twice with ice cold HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose, pH 7.4; HBS), then incubated in HBS with[^3H]folic acid (50 nM, specific activity 0.5 Ci/mmol) in the presence and absence of a range of concentrations of unlabeled folic acid or antifolate for 15 min at 0 °C. The dishes were rinsed three times with ice-cold HBS, after which the cells were solubilized with 0.5 N sodium hydroxide and aliquots measured for radioactivity and protein contents. Protein concentrations were measured with
Folin phenol reagent. Bound $[^3]$Hfolic acid was calculated as pmol/mg protein. Relative binding affinities for assorted folate/antifolate substrates were calculated as the inverse molar ratios of unlabeled ligands required to inhibit $[^3]$Hfolic acid binding by 50%. By definition, the relative affinity of folic acid is 1.

**PCFT and RFC Transport Assays.** For transport assays, R2/hPCFT4, PC43-10, and R2(VC) CHO cells grown as monolayers were used to seed spinner flasks. For experiments to determine the inhibitions of transport by antifolate substrates, cells were collected and washed with DPBS and resuspended in 2 mL of physiologic Hank’s balanced salts solution (HBSS) for PC43-10 cells and in HBS adjusted to pH 7.2 or 6.8 or 4-morpholinesulfonic acid (MES)-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, and 5 mM glucose) adjusted to pH 6.5, 6.0, or 5.5 for R2/hPCFT4 cells. In either case, uptakes of $[^3]$HMTX (0.5 μM) were measured over 2 min at 37 °C in the presence and absence of unlabeled antifolates (10 μM). Uptakes of $[^3]$HMTX were quenched with ice-cold DPBS. Cells were washed with ice cold DPBS (3×) and solubilized with 0.5 N NaOH. Levels of intracellular radioactivity were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Protein concentrations were measured with Folin phenol reagent. Percent MTX transport inhibition was calculated by comparing level of $[^3]$HMTX uptake in the presence and absence of the inhibitors. Kinetic constants (K$_t$, Vmax) and K$_i$s were calculated from Lineweaver-Burke and Dixon plots, respectively.

**Protection experiments.** IGROV1 cells were plated (2000 cells/well) in folate-free RPMI 1640 medium with 10% dialyzed FBS, antibiotics, l-glutamine, and 25 nM LCV with a range of drug concentrations in the presence of adenosine (60 μM), thymidine (10 μM), AICA (320 μM) or glycine (130 μM). Cell proliferation was assayed with Cell Titer Blue
(Promega) using a fluorescence plate reader. Data are representative of at least triplicate experiments. Error bars represent the standard errors.
Uncategorized References


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