Discovery of Pyrimidine-based Heterocycles as Single Agents With Combination Chemotherapy Potential And As Inhibitors Of Purine Nucleotide Biosynthesis For The Treatment of Cancer

Rishabh Mohan

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DISCOVERY OF PYRIMIDINE-BASED HETEROCYCLES AS SINGLE AGENTS WITH COMBINATION CHEMOTHERAPY POTENTIAL AND AS INHIBITORS OF PURINE NUCLEOTIDE BIOSYNTHESIS FOR THE TREATMENT OF CANCER

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Submitted to the Graduate School of Pharmaceutical Sciences

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

By
Rishabh Mohan

December 2017
DISCOVERY OF PYRIMIDINE-BASED HETEROCYCLES AS SINGLE AGENTS
WITH COMBINATION CHEMOTHERAPY POTENTIAL AND AS INHIBITORS OF
PURINE NUCLEOTIDE BIOSYNTHESIS FOR THE TREATMENT OF CANCER

By

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Approved July 19, 2017

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By

Rishabh Mohan

December 2017

Dissertation supervised by Professor Aleem Gangjee

This dissertation describes the design, synthesis and biological evaluation of monocyclic and bicyclic pyrimidine-base heterocycles as single agents with combination chemotherapy potential having both antiangiogenic effects and cytotoxic effects. This dissertation also describes selective tumor targeting with 5-substituted pyrrolo[2,3-\textit{d}]pyrimidines analogs with heteroatom bridge substitution as GARFTase inhibitors.

The work in this dissertation is centered on identifying structural features that are necessary for inhibition of tubulin polymerization as well as for inhibition of one or more of the receptor tyrosine kinases (RTKs)- vascular endothelial growth factor receptor-2 (VEGFR2), platelet derived growth factor receptor-\(\beta\) (PDGFR\(\beta\)) and epidermal growth factor receptor (EGFR) in single entities. Single agents with both antiangiogenic activities as well as cytotoxicity 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.

This work reviews the synthesis of substituted monocyclic pyrimidines as well as pyrrolo[2, 3-\textit{d}]pyrimidines. This work also reviews the synthesis of multi-transporter (PCFT and FR) selective 5-substituted pyrrolo[2, 3-\textit{d}]pyrimidines as GARFTase inhibitors circumventing both dose-limiting toxicity and tumor resistance associated with most prescribed antitumor agents like pemetrexed.
ACKNOWLEDGEMENTS

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<td>ATP binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BP</td>
<td>back pocket</td>
</tr>
<tr>
<td>CA4</td>
<td>combretastatin A4</td>
</tr>
<tr>
<td>CA4P</td>
<td>combretastatin A4 phosphate</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>DFG</td>
<td>aspartate-phenylalanine-glycine</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
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<tr>
<td>EGF</td>
<td>endothelial growth factor</td>
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<td>EGFR</td>
<td>endothelial growth factor receptor</td>
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<td>GDP</td>
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<td>MAP</td>
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<td>MDR</td>
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<td>platelet derived growth factor</td>
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<tr>
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I. BIOLOGICAL REVIEW

A. Microtubules

Cytoskeletal protein polymers which include microfilaments (actin proteins), intermediate filaments (various proteins e.g. desmin, peripherin, keratin) and microtubules (tubulin proteins) are crucial for cytoskeletal function and provide mechanical support and resistance against physical stress. Microtubule targeting agents (MTAs) affect interphase cells significantly by disrupting transcription, translation, axonal transport, mitochondrial permeability, immune cell function, directional migration and centrosome clustering (Figure 1).1 In interphase cells, MTA treatment perturbs microtubule dynamicity as well as significantly disturbs interphase intracellular trafficking of protein and nucleic acid cargo in various types of cancer cells.2 For this reason, MTAs that interfere with these processes possess potent inhibitory activities against a wide variety of tumors.3 Drugs targeting microtubules have been highly successful in the clinic and have been integrated into therapeutic regimens against both solid tumors (e.g., breast, ovarian, non-small-cell lung cancer, and Kaposi’s sarcoma) and hematological malignancies (non-Hodgkin's lymphomas).4,5

Microtubules are assembled in polarized arrays during the G1-, S-, and G2-phases (Figure 2)2 with their (+) end pointing outward from the center and the (−) end is nucleated by the centrosome. These move to the two opposite sides of the cell during the mitosis phase, forming the mitotic spindle.
A.1 Structures of Microtubules

Microtubule/tubulin is one of the most important and promising targets in cancer therapy. Microtubules are tube-like protein filamentous fibers made of individual tubulin dimers. The tubulin heterodimer consists of one α- and one β-tubulin subunit (molecular weight = 50 kDa). Tubulin dimers polymerize in a “head-to-tail” fashion between the α- and β-tubulin to form protofilaments which further arrange in parallel to form a hollow cylindrical microtubule.

Figure 1. Interphase activities of MTAs (modified from ref. 1)
(a) Centrosome clustering. (b) voltage-dependent anion channel opening (c) disruption of delivery of mRNA along interphase microtubule tracks to polysomes and (d) induce mRNA release from polysomes. (e) improve MHC class I expression by cancer cells (f) activation of dendritic cells, (g) cytotoxic T lymphocytes and (h) macrophages (i) impede vesicular traffic to the cell front (j) timely endocytosis of focal adhesion components (k) interference with transcription factor transport (l) up- or down-regulation of tumor suppressor or oncogenes, respectively.

Figure 2. Microtubules organization during the cell cycle (modified from ref. 2).
structure (Figure 3). Each α- and β-tubulin heterodimer structure includes two GTP molecules, one of which is tightly bound and the other bound GTP molecule is freely exchangeable with unbound GTP. The non-exchangeable GTP is bound to α-tubulin, whereas GTP and GDP are exchangeable on β-tubulin. GTP which is exchangeable is highly involved in the regulation of various functions of tubulin. Hydrolysis of GTP is necessary for microtubule depolymerization and binding of GTP is required for microtubule polymerization.⁷

**Figure 3.** A. Cervical cancer HeLa cell with individual microtubule filaments stained green and nucleus stained red. B. and C. arrangement of 13 protofilaments. D. atomic-
resolution model of a 13 protofilament microtubule. E. apical side view of this model (modified from ref. 7)

Microtubule associated proteins (MAPs) impact protein–microtubule interactions, microtubule cross-linking and overall microtubule stability. The fast growing plus end probes the cytoplasm in a search-and-capture process to reach different targets but the slow growing minus end is anchored at the microtubule organizing center. 3

A.2 Microtubule Dynamics

Microtubule dynamics are responsible for the functional role of microtubules in cells and are very tightly regulated. 3 MAPs, kinases, phosphatases and nucleotides regulate these dynamics. Microtubule dynamics, if disturbed, can have serious consequences on the fate of a cell and therefore, disruption of microtubule dynamics is a very important strategy to develop anticancer agents. 8 Tubulin polymerization involves slow formation of a short microtubule nucleus which is followed by a rapid phase of microtubule elongation through reversible and non-covalent addition of αβ-tubulin heterodimers. GTP binding to β-tubulin favors a dimer conformation that promotes polymerization. 9 Upon the attachment of the GTP-bound tubulin dimer to the ends of the microtubule, GTP hydrolysis occurs, which further drives polymerization forming tubulin–GDP with the release of P_i which provides energy for the addition of a dimer.
The presence of a GTP cap i.e. GTP or GDP with unreleased phosphate (GDP-P$_i$). at the end of the microtubule, stabilizes the open-sheet conformation of the growing microtubule plus end, and thus lengthens the microtubule. The GTP cap prevents shrinkage of the microtubule and the microtubule end shortens rapidly after loss of the GTP cap.$^{10}$

Two major forms of non-equilibrium dynamics occur in vitro and in cells, namely dynamic instability and treadmilling (Figure 4).$^4$ Dynamic instability (Figure 5) is the switching of microtubules between phases of lengthening and shortening, and is defined by some factors:$^9,^{11}$ 1) the rate of growth and shrinkage 2) The transition from a growth or a pause state to a shortening phase (“catastrophe”) 3) The transition from a shortening phase to a
growth or a pause state ("rescue"). Another kind of microtubule dynamics behavior is treadmilling (Figure 6). This process is characterized by net growth at one end and balanced equivalent shortening at the opposite end.

**Figure 5.** Microtubule growth and shrinkage (modified from ref. \(^{12}\))

**Figure 6.** Microtubule treadmilling (modified from ref. \(^{13}\))
A.3 Microtubule Binding Agents: On the basis of their effects on microtubule stability they are classified as follows.\textsuperscript{7,14}

1) \textit{Microtubule-destabilizing agents} include natural products such as the Vinca alkaloids, estramustine, colchicine and its analogs and combretastatin. They inhibit microtubule polymerization and so, promote microtubule disassembly.\textsuperscript{7}

2) \textit{Microtubule-stabilizing agents} include paclitaxel, docetaxel, epothilones and discodermolide. These agents induce microtubule polymerization and so, promote microtubule assembly.

Microtubule binding agents can also be classified by their binding sites on tubulin.\textsuperscript{14}

1) \textit{The Vinca alkaloids} including vinblastine, vincristine, vindesine, and vinorelbine and bind to the $\beta$-subunit of soluble tubulin heterodimers (Figure 7)\textsuperscript{15} and have been widely used in cancer chemotherapy for the treatment of leukemias, lymphomas, small cell lung cancer, and other cancers. The rapid and reversible binding of vinblastine to soluble tubulin induces a conformational change that prevents integration of the dimer into the microtubule.\textsuperscript{16}
2) Taxanes and Epothilones

Figure 7. Vinca binding site and representative vinca alkaloids (modified from ref. 14)

**Figure 8.** Taxane binding site and representative taxanes (modified from ref. 14)
The second class of antimitotics, the taxanes, are exemplified by paclitaxel and docetaxel which bind poorly to soluble tubulin, but bind strongly to tubulin along the length of the microtubule.\textsuperscript{17} The binding site for paclitaxel is on the inside surface of the β-subunit of the microtubule (Figure 8).\textsuperscript{15, 18} Paclitaxel induces an increase in microtubule polymerization, thereby interfering with spindle microtubule dynamics and are termed as microtubule-stabilizing agents or polymerizing agents.\textsuperscript{19} The taxanes are widely used for breast cancer, ovarian cancer, non-small cell lung cancer, and Kaposi’s sarcoma. Ixabepilone was approved for the treatment of drug-refractory metastatic breast cancer in 2007.\textsuperscript{18}

3) Colchicine binding site agents

The third class is typified by colchicine binding site agents include podophyllotoxin, combretastatins and flavonols. Colchicine produces toxicity at doses that produce antimitotic effects, and so, is not used clinically in cancer treatment.\textsuperscript{20} The colchicine binding site is on β-tubulin at the interface between α- and β- tubulins (Figure 9).\textsuperscript{15}

![Figure 9. Colchicine binding site and combretastatins (modified from ref. \textsuperscript{14})](image_url)
Combretastain A4 (CA4) is a potent vascular-disrupting agents (VDA) in addition to microtubule targeting agent (MTAs).\textsuperscript{21-22} CA4 and CA4P have been developed as antivascular agents for clinical trials. Combretastatins also inhibit angiogenesis, and so, have been used to target target tumor vasculature selectively as an alternative to the conventional chemotherapy.\textsuperscript{7}

4. Additional binding sites: Several MTAs such as discodermolide,\textsuperscript{23} noscapine,\textsuperscript{24} laulimalide,\textsuperscript{25} and estramustine\textsuperscript{7} show potent microtubule inhibitory effects but do not bind to the above mentioned three sites on tubulin.\textsuperscript{15, 18}

A.4 Resistances to microtubule-binding agents- overexpression of Pgp and increased levels of βIII-tubulin.

Multiple-drug resistance (MDR) is a one of the biggest causes of cancer chemotherapy failure, which includes the failure of microtubule-binding agents.\textsuperscript{5, 17, 26} Among different resistance mechanisms for this category of drugs, the most clinically significant are structural alteration of the microtubules and drug efflux by ATP binding cassette (ABC) proteins.\textsuperscript{27-28} The increased efflux of hydrophobic cytotoxic drugs that is mediated by one family of energy-dependent transporters (e.g., P-glycoprotein (Pgp)).\textsuperscript{29} In Pgp-overexpressing SK-OV-3 MDR-1-6/6 cell lines, it is observed that the potency of paclitaxel was reduced by 800-fold versus parental cell lines.\textsuperscript{30} Interestingly, the combination of microtubule-binding agents with Pgp inhibitors failed to reverse resistance in clinical trials.\textsuperscript{31} Another concern is increased levels of βIII-tubulin which is a major cause of clinical resistance against taxanes in lung, breast and ovarian cancers.\textsuperscript{32-33} The potency of
paclitaxel decreases by five-fold in HeLa cells when they were adapted to express βIII-tubulin. βIII-tubulin also causes resistance against a wide range of drugs with different mechanisms of action.

**B. Angiogenesis**

Angiogenesis is the process of formation of new blood vessels from existing vascular beds. It involves a complex cascade of cellular events that are tightly regulated by proangiogenic factors including several growth factors and antiangiogenic factors. An upregulated angiogenesis has been observed as one of the hallmarks of cancer, playing an essential role in tumor growth, invasion, and metastasis.

During the early stages of development of a tumor, blood supply to the tumor tissue comes from the vasculature supplying the surrounding normal healthy cells. But, as the tumor grows beyond 2 mm in diameter, it requires its own blood supply to support its growth. It becomes increasingly hypoxic, and triggers the release of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet derived growth factor (PDGF) among others. These growth factors act as proangiogenic signals to initiate angiogenesis. (Figure 10). The new blood vessels grow and infiltrate into the tumor which results in tumor progression and metastases. The sustained growth of solid tumors is highly dependent on angiogenesis. Thus, antiangiogenesis is a potential approach and has been widely used in the treatment of cancer.

The growth of tumor tissue vasculature is chaotic and leaky. Therefore, tumor vasculature differs from normal tissue vasculature in several aspects including the hierarchy of organization and nature of endothelium.
Figure 10. The angiogenic process (modified from ref. 45)

**B.1 Receptor Tyrosine Kinases (RTKs):**

The pro-angiogenic growth factors bind to transmembrane receptors of the protein kinase family identified as RTKs, and initiate signal transduction which initiates angiogenesis. These RTKs, namely vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor (PGFR) consist of an extracellular domain, a transmembrane domain and an intracellular kinase catalytic domain. 46 Binding of the growth factor to the extracellular domain results in receptor homo
or heterodimerization and autophosphorylation of tyrosine residues within the catalytic domain.

**Figure 11.** Representative RTK inhibitors approved for clinical use in USA.

This triggers a cascade of events through phosphorylation of intracellular proteins that ultimately transmit the extracellular signal to the nucleus, eventually leading to angiogenesis. Dysregulation of these signaling pathways has been linked to malignancy and is designated as a hallmark of cancer.\(^{47}\) Inhibition of these RTKs offers a key therapeutic strategy for targeted cancer therapy.\(^{48}\) Several RTKs targeting small molecular inhibitors (Figure 11) including erlotinib, imatinib, gefitinib, sorafenib, sunitinib have
been approved as "targeted agents" for the clinical treatment of various types of cancer.48-49

A number of RTKs have been recognized to be involved in tumor induced angiogenesis. The key RTK families in cancer include the platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and the VEGFR families of RTKs

**VEGFR**

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). Three types of VEGFRs have been identified, VEGFR1, VEGFR2 and VEGFR3. VEGFR-2 (Flk-1/KDR) has been recognized as the principal receptor that mediates VEGF stimulation which is one of the most important growth factors that is involved in angiogenesis and vasculogenesis.50 Targeted VEGFR2 inhibition or disruption of produces an abrogation of angiogenesis, and decreased tumor growth.50-51 Sunitinib and semaxanib are some of the inhibitors of VEGFR2 which have displayed antiangiogenic activity.42, 52

**EGFR**

The EGFR family of receptors contain four members: EGFR-1, also identified as human EGF receptor HER-1), HER-2, HER-3, and HER-4. Among these, HER-2 lacks a known endogeneous ligand and HER-3 lacks kinase activity. The EGF family is comprised of eleven known members that bind to one of these four EGFR family receptors.53 Overexpression of EGFR, and its growth factors lead to increased tumor cell proliferation, survival and invasiveness.54 EGFR signaling if inhibited, could promote selective apoptosis in tumor endothelial cells.55 Abnormal activity through overexpression or constitutive
action, has been linked to a number of cancers, including prostate, lung, and breast cancers.\textsuperscript{56}

**PDGFR**

It is comprised of four structurally related polypeptides namely -AA, -BB, -CC, -DD, and -AB that exist as five different homodimers and heterodimer ligands.\textsuperscript{57} PDGF can bind to and activate two of its receptors PDGFR\(\alpha\) and PDGFR\(\beta\)\textsuperscript{54} which are expressed on pericytes, smooth muscle cells and provide mechanical support to vasculature. By stimulating the formation of microvascular pericytes, and stabilizing the newly formed blood vessels, PDGFR plays an important role in angiogenesis.\textsuperscript{38}

**B.2 ATP Binding Site of RTKs**

The site that has been has been indicated as a promising target for rational drug design is the ATP binding site of RTKs. Diversity and homology among the ATP-binding sites of kinases has allowed the building of pharmacophore models for rational drug design in cancer chemotherapy.\textsuperscript{58-59} A twisted \(\beta\)-sheet of five antiparallel \(\beta\)-strands and one \(\alpha\)-helix make up the N-terminal lobe. The C-terminal lobe is made up of four \(\beta\)-strands and eight \(\alpha\)-helices.
Figure 12. ATP-binding site of RTKs (modified from ref. 60)

**RTK Catalytic Cleft:** The catalytic cleft consists of two regions, the front cleft and the back cleft. The front cleft of the catalytic domain is occupied by the ATP-binding site, while the back cleft is comprised of elements responsible for the regulation of phosphorylation of peptide substrates.

A. **The Front Cleft:** The front cleft consists of the ATP binding site and small, non-ATP contact regions. The ATP site is divided into the following subregions depending on the binding mode (Figure 12).  

1. Adenine region: This region is predominantly hydrophobic and is bordered by the hinge region and the gatekeeper. Two key hydrogen bonds are formed by the interaction of the N1 and N6 amino nitrogens of the adenine ring with the NH and carbonyl groups of the peptide backbone of the hinge region residues of the RTKs.

2. Ribose binding region: This pocket is adjacent to a hydrophilic, solvent exposed region and accommodates the sugar moiety of ATP. The ribose pocket accommodates the sugar
moiety of ATP and is adjacent to a hydrophilic, solvent exposed region. This pocket includes three hydrophobic and hydrophilic residues. These hydrophobic residues (Ile, Val and Leu) have traditionally been exploited in developing EGFR inhibitors. Our group has published many papers exploiting the hydrophobic residues of the sugar binding pocket of the ATP site.

3. Phosphate binding region: This pocket is flexible, hydrophilic and solvent exposed, and is covered by Asp, Lys and Asn residues and the DFG motif. Being highly flexible and solvent exposed, this region is considered less important in improving inhibitor affinity and potency.

4. Hydrophobic region II: This pocket serves as an entrance for ligand binding as it is not utilized by ATP, and so variation in the residues and conformation of this pocket has been reported for various kinase targets. To gain selectivity in the design of kinase inhibitors, this region has highly ben been used.

B. The Back Cleft: The back cleft provides important binding regions for various kinase inhibitors. ATP does not bind in the pockets in the back cleft. The hydrophobic pocket in the back cleft adjacent to the adenine pocket is called Hydrophobic region I. This pocket has highly been explored in the design of inhibitors to gain selectivity for kinase targets with small gatekeeper residues.
C. Combination Chemotherapy

In order to bypass inhibition of a single biological target, cancer cells often find alternative compensatory signaling routes. These findings provide the rationale for the use of drugs able to target multiple unrelated proteins. It is not surprising that this strategy has found utility in complex disease states such as cancer with remarkable clinical success. The concept of using a combination of drugs each with activity against a different biological target has been used in the treatment of several diseases. The central idea of combination chemotherapy is that by modulating multiple pathways simultaneously, the utilization of alternate survival pathways that allow tumor cells to evade the therapy can be prevented.

Antiangiogenic RTK inhibitors are cytostatic agents that prevent further growth of the tumor and are required to be used in combination with cytotoxic agents for complete abrogation of tumors. Of significant interest is the remarkable clinical success of combinations of antiangiogenic agents and cytotoxic agents. Sorafenib (5 µM) significantly enhanced the anti-cancer effect (25% growth inhibition compared to control) of low dose paclitaxel (125 pM) in microvascular endothelial cells, producing an additive effect on inhibition of cell growth.
D. Vascular Normalization Theory

Antiangiogenic agents target endothelial cell-mediated angiogenesis, and indirectly stop growth of tumor cells by depriving them of nutrients, while cytotoxic agents kill cancer cells directly. However, destroying the tissue vasculature is expected to severely compromise the delivery of the cytotoxic agent to the solid tumor and render these compounds inactive. Surprisingly, synergistic antitumor effects via the use of antiangiogenic and cytotoxic drug combinations lead to the proposal of the "vascular normalization" theory.70-71

Under healthy physiological conditions, a balance between endogenous proangiogenic and antiangiogenic factors maintains normal vessels. These vessels are arranged in a hierarchical manner (i.e., arteries, capillaries, and veins) forming an orderly distributed compact network, whereas tumor vasculature is disorganized and morphologically abnormal with a leaky endothelium. In tumor environments, an abundance of proangiogenic growth factors results in vascular growth being vigorous and random in all possible directions at all junctures. Consequently, the difference in pressure between the arterioles and venules is reduced, resistance to blood flow is increased, and blood supply to the tumor is impaired.72 The vascular network appears haphazard with high vascular density in certain regions of the tumor and lack of vasculature in others.73 Inefficient tumor blood flow due to abnormal vasculature can negatively affect the delivery of cytotoxic drugs and also cause systemic toxicities. However, as the targets of antiangiogenic agents
are located in contact with the bloodstream, these drugs are considered to suffer less from delivery obstacles than drugs targeting tumor cells in the extravascular space.

Figure 13. Antiangiogenic therapy and vessel normalization (modified from ref. 70)

The effect of antiangiogenic agents is twofold: first, they prune some of the abnormal vessels and second, due to their antiangiogenic nature, these agents can balance the effect of proangiogenic factors and result in a transient normalized vasculature (Figure 13). This transient normalization is both structural and functional. The normalized vasculature is central for blood flow with the least obstruction and blood supply to the tumor. As a result, there is improved delivery of the cytotoxic agent to the tumor thereby explaining, in part, the synergy observed in the use of antiangiogenic and cytotoxic drug combinations.

Anti-VEGF treatment induces a transient normalization process that leads to pruning of immature vessels, and improvement of the integrity and function of the remaining
vasculature by enhancing perivascular cell and basement membrane coverage.\textsuperscript{74-75} Normalization of tumor vasculature increases uptake and delivery of cytotoxic antitumor drugs.\textsuperscript{76-77} However, enhancement in the delivery of cytotoxic agents to the tumor is both dose- and time-dependent. The cytotoxic agent should be delivered to the tumor during the "window of normalization" in order to maximize the intratumoral cytotoxic drug levels. As a result, optimal scheduling of antiangiogenic therapy with chemotherapy and/or radiation therapy requires knowledge of the timing window during which the vessels transiently become normalized, and an understanding of the duration of the normalized vasculature. Improper understanding could cause the chemotherapeutic agent to miss the therapeutic window, and suboptimal effects or no effect of combination chemotherapy could result.

E. Multiple target inhibition with single agents

Multitargeted drugs are defined as rationally designed single chemical entities capable of selectively targeting two or more biological targets or processes.\textsuperscript{78} If the pharmacophores for the respective targets overlap, the common structural features of the lead compounds can be "merged" resulting in a designed multiple ligand. As with combination chemotherapy, targeting different pathways in tumor cells using multitargeted agents can increase therapeutic effectiveness, as this strategy may prevent cancer cells from developing resistance, which is the principal cause of chemotherapy failure.\textsuperscript{79} Other advantages include a lower risk of drug-drug interactions \textit{in vivo} and improved patient compliance resulting from reduction in the number of medications required. At the level of biochemical response, a single agent with multiple targets would be less prone to interact
with anti-targets compared to multiple separate agents which would be more prone to produce off-target toxicities. So, a single agent would have less adverse effects, if any, compared to multiple agents. Optimizing the pharmacokinetics of the lead compound while retaining a balanced target profile (i.e., optimizing the ratio of activities at the different targets and the effects of metabolism to ensure a therapeutic, but non-toxic, effect at each of these targets) is one of the challenges of the multitargeted agents. Several multitarget strategies have been studied in cancer therapy. A clinical strategy that has been successful is the combination of antiangiogenic and cytotoxic agents.

F. Folic acid (FA)

A p-aminobenzoic acid (PABA), a hetero-bicyclic pteridine, and a glutamic acid are the three structural units of folic acid (FA) (Figure 14). FA, an oxidized synthetic compound is used in dietary supplements. The word folate refers to the various reduced derivatives of FA naturally found in food. Folates occur in several reduced states of the pteridine ring (oxidized, 7,8-H2, and 5,6,7,8-H₄).

![Figure 14. Structure of folic acid with the three principal moieties of the molecule.](image-url)
1. **Folate metabolism:**

Folic acid metabolism cycle in mammals is a crucial process for the transfer of one-carbon units to amino acids, nucleotides, and other biomolecules. The proton coupled folate transporter (PCFT) in the intestines plays a major role for the folic acid uptake. Folates have to be reduced to tetrahydrofolate (THF) to act as a cofactor. The first step is the intracellular reduction of the folic acid, in presence of reductant nicotinamide adenine dinucleotide phosphate (NADPH) to the intermediate 7,8-dihydrofolate (DHF). This is followed by a similar reduction of DHF to THF.

![Diagram of de novo synthesis of purines](image)

**Figure 15.** *De novo* synthesis of purines
β-Glycinamide-ribonucleotide transformylase (GARFTase) and amino-imidazolecarboxamide ribonucleotide transformylase (AICARFTase) are two folate related enzymes required for the de novo synthesis of purines, that utilize the cofactor $N^{10}$-formyltetrahydrofolate ($N^{10}$-CHO-THF) to transfer one carbon units (Figure 15). $N^{10}$-CHO-THF and glycinamide ribosyl-5-phosphate (GAR) are converted to THF and formyl-glycinamide ribosyl-5-phosphate (fGAR) respectively in the presence of glycinamide-ribonucleotide formyl transferase (GART). The fGAR formed is then converted to amino-imidazolecarboxamide ribosyl-5-phosphate (AICAR) in five steps. The enzyme AICARFTase then transforms AICAR to formyl-AICAR (fAICAR). THF is also released in this process. The term antifolates is a class of antiproliferatives that closely resemble FA, and so, interfere with the biosynthesis, utilization or metabolism of normal cellular folate cofactors involved in folate metabolism.\(^8\)

The fAICAR formed continues along the purine biosynthetic pathway leading to the formation of inosine-5’-monophosphate (IMP), the precursor of adenosine-5’-triphosphate (ATP) and guanosine-5’-triphosphate (GTP) necessary for ribonucleic acid (RNA) synthesis and of 2’-deoxyadenosine-5’-triphosphate (dATP) and 2’-deoxyguanosine-5’-triphosphate (dGTP) necessary for DNA synthesis.\(^7\)

Folates are hydrophilic anionic molecules, and so, cannot passively diffuse across biological membranes. Several sophisticated membrane transport systems are evolved in mammals to facilitate membrane translocation of these essential cofactors. A folate molecule is transported into the cell in its monoglutamate form and then, is metabolized by folyl-γ-glutamate synthetase (FPGS) by adding L-glutamic acid units to the γ-carboxylic
acid (up to eight glutamate moieties). The polyglutamate cofactor form is termed folypoly-γ-glutamate and this process is described as polyglutamylation. This leads to (i) increased intracellular retention of folates through metabolic trapping; (ii) enhanced binding of polyglutamylated tetrahydrofolate (THF)

\[\text{Methotrexate } R = \text{CH}_3 \text{ (DHFR)}\]
\[\text{Aminopterin } R = \text{H} \text{ (DHFR)}\]
\[\text{Pemetrexed (TS)} \text{ (LY231514, Alimta)}\]
\[\text{Pralatrexate (DHFR)}\]
\[\text{Ralitrexed (TS)} \text{ (ZD1694, Tomudex)}\]
\[\text{Plevitrexed (TS)} \text{ (ZD9331)}\]
\[\text{GW1843U89 (TS)}\]
\[\text{PDDF (TS)}\]
\[\text{Lometrexol (GARFTase)}\]

**Figure 16.** Representative examples of classical antifolates with principal targets.

to folate-dependent enzymes at low concentrations; (iii) increased retention of folate cofactors in mitochondria. γ-Glutamyl hydrolase (GGH), present in the lysosome, catalyzes the hydrolysis of polyglutamates back to their monoglutamate form. Antifolates which contain L-glutamic acid at the side chain such as methotrexate (MTX),
aminopterin, pemetrexed (PMX, Alimta), raltitrexed (RTX, ZD1694, Tomudex), pralatrexate (PDX) (Folotyn, 10-propargyl-10-deazaaminopterin, N-(4-((1-(2,4-diamino-6-pteridinyl)methyl)-3-butynyl)benzoyl)-L-glutamic acid), lometrexol etc. are known as classical antifolates (Figure 16).

2. Membrane transport of folates: Mammalian cells have evolved genetically distinct systems to facilitate membrane transport of folates. Three major folate uptake systems, the reduced folate carrier (RFC), folate receptor (FRs) and the proton-coupled folate transporter (PCFT) have been described. Each plays a unique role in mediating folate transport across epithelia and into tissues.

2.1. The Reduced Folate Carrier (RFC)

RFC is the major cellular and tissue folate transporter in mammalian cells. It is ubiquitously expressed in mammals. RFC is also a major transporter of antifolate drugs used in therapy for several illnesses and for cancer chemotherapy. The effectiveness of chemotherapy with agents such as MTX, PMX, and RTX (Figure 18) is closely linked to levels and activity of RFC in both tumors and normal tissues.

2.1.1. Structure of RFC

RFC is the major membrane transporter of circulating folate cofactors and belongs to the major facilitator superfamily of hRFC. It is comprised of 591 amino acids and is 64-66% conserved with rodent RFC. RFC is characterized by 12 transmembrane domains
(TMDs) and is N-glycosylated at Asn58 in the extraloop domain connecting TMDs 1 and 2. A large loop domain connects TMD6 and TMD7, and is poorly conserved between species. The major role for the TMD6–7 loop domain is to provide appropriate spacing between the TMD1–6 and TMD7–12 segments for optimal membrane transport.\textsuperscript{88}

2.1.2. RFC distribution

In liver and placenta, high levels of RFC transcripts have been detected, with significant levels in other tissues, including leukocytes, kidneys, lungs, bone marrow, intestines, and portions of the CNS and brain. RFC was also detected at the basolateral membrane of the renal tubule epithelium, the apical brush-border membrane of the small intestine and colon, hepatocyte membranes, the apical surface of the choroid plexus, and the apical membrane of the cells lining the spinal canal.\textsuperscript{95} Low levels of RFC result in pathologic conditions such as cardiovascular disease, fetal abnormalities, neurological disorders, and possibly cancer.\textsuperscript{96} RFC is important for development since inactivating both RFC alleles in mice by targeted homologous recombination is embryonic lethal.\textsuperscript{97}

2.1.3. Transport mechanism of RFC

RFC transports reduced folates via counter-transport with organic phosphate anions. The major circulating form of folate, 5-methylTHF, is its physiological substrate. RFC has high affinity (~50-100-fold) for reduced folates ($K_i \approx 1-5 \, \mu\text{M}$) and a low affinity for folic acid ($K_i \approx 200 \, \mu\text{M}$) as it is an integral transmembrane protein.\textsuperscript{98} RFC transport is characterized by a neutral pH optimum and so, it has significantly decreased transport
activity below pH 7. As folates are negatively charged, RFC actually produces a substantial electrochemical potential difference for folates across cell membranes.\(^9^9\) RFC-mediated transport is highly sensitive to the transmembrane anion gradient, in particular, the organic phosphate gradient. So, asymmetrical distribution of organic phosphates across cell membranes provides the driving force for RFC-mediated uphill transport of folates into cells.\(^9^7\)

### 2.1.4. RFC in antifolate chemotherapy

The classical antifolates MTX, RTX, and PMX (Figure 16) are all actively transported into mammalian cells by RFC.\(^1^0^0\) However, RFC is ubiquitously expressed in mammalian cells and, is a major folate transporter for normal tissues and tumors. These elevated extracellular antifolate concentrations always result in dose limiting toxicity due to a lack of RFC transport selectivity for tumor cells over normal cells.

### 2.2. The folate receptors (FRs)

The FRs represent another mode of folate uptake into mammalian cells, and are a family of high affinity folate binding proteins.

#### 2.2.1. Structure of FRs

The FRs are encoded by three distinct genes, designated \(\alpha\) and \(\beta\) localized to chromosome 11q13.3-q13.5.\(^1^0^1\) Another FR gene, designated \(\gamma\), maps at chromosome 11q14.\(^1^0^2\) FRs \(\alpha\), \(\beta\) and \(\gamma\) are highly homologous proteins (68-79% identical amino acid
sequence) and contain from 229 to 236 amino acids with two (β, γ) or three (α) N-glycosylation sites. In contrast to RFC, hFRα and hFRβ are anchored at the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchors, whereas FRγ is secreted due to the lack of a signal sequence for GPI anchor attachment.

**Figure 17.** a. Two views of the FRα-folic acid complex, with FRα in green, folic acid in grey, N-acetylglucosamine in orange and the disulphide bonds depicted as yellow sticks.(modified from ref.103)

Recently, the crystal structures of both FRα and FRβ were published. The crystal structure of FRα with folic acid at 2.8 Å resolution was reported by Karsten Melcher and co-workers (Figure 17).103 FRα has a globular structure stabilized by eight di FRα has an overall globular structure, consisting of four long α-helices (α1, α2, α3, α6), two short α-helices
(α4, α5), four short β-strands (β1–β4) and multiple loop regions. The tertiary structure of FRα is stabilized by eight disulphide bonds formed by 16 conserved cysteine residues. It also contains a deep open folate-binding pocket comprised of residues that are conserved in all folate receptor subtypes.\textsuperscript{103}

Charles E. Dann III and coworkers\textsuperscript{104} reported six different conformations of FRβ at neutral and acidic pH. The results suggest that the pH of the receptor environment plays a crucial role in the rearrangements of the structure that at neutral pH, folate binding cleft is poised in an open conformation and at the acidic pH which is experienced in the endosome, it is occluded which means that transport is most efficient at neutral pH and least efficient at acidic pH.\textsuperscript{104}

\textbf{2.2.2. FRs distribution}

FRα is generally localized on the apical surface of polarized epithelial cells, particularly in the proximal tubule cells of the kidney, choroid plexus, retina, uterus and placenta. FRα is not in contact with circulating folate as it is predominantly expressed on the apical surface. This unusual polarized expression of hFRα protects normal tissues from FR-targeted therapies in the blood circulation. hFRβ is expressed in the placenta, spleen, and thymus and in the latter stages of normal myelopoiesis. hFRγ is secreted from lymphoid cells in the spleen, thymus, and bone marrow at low levels. The restricted distribution of FRs, the transcytosis mechanism used by hFRα, and the generally low expression levels of hFRβ play a crucial role in limiting toxicities in normal tissues with
FR-targeted therapeutics. Therefore, such agents circumvent the dose limiting toxicities exhibited by currently used non-selective antifolates, that are also transported by RFC.\textsuperscript{105}

### 2.2.3. Transport mechanism of FRs

![Diagram of FR transport mechanism](image)

**Figure 18.** Endocytosis of FRs.

When a folate molecule binds to a folate receptor on the cell surface, the process of internalization by FRs is initiated, which leads to conformational change and invagination of the plasma membrane at that site followed by the formation of vesicles (endosome) that migrate to the cytoplasm. This process is known as endocytosis (Figure 18). The interior of endosome becomes acidic (pH = 6.0-6.5) once it enters the cytoplasm. This decrease in pH leads to another conformational change of the FR complex resulting in the dissociation
of the folate from the complex. The endosome then migrates back to the cell surface, gets fused with the cell membrane and the same FRs is recycled.¹⁰⁶-¹⁰⁷

2.2.4. FRs and antifolate chemotherapy

FRα is overexpressed in up to 90% of ovarian cancers.¹⁰⁸-¹⁰⁹ Unlike tumor cells, FRα in normal tissues is inaccessible to the blood circulation.¹¹⁰ FRβ is expressed in a wide range of myeloid leukemia cells. FRβ in normal hematopoietic cells differs from that in leukemia cells in its inability to bind folate ligand.¹¹¹ Unfortunately, for most classical folate-based therapeutics (including MTX, RTX and PMX), since substrates are shared between FRs and the ubiquitously expressed RFC, tumor selectivity is not present. That is why, if a FR-targeted ligand were itself cytotoxic without RFC activity, selective tumor targeting would ensue.¹¹²

2.3. The proton-coupled folate transporter (PCFT)

PCFT is a member of the superfamily of solute transporters. The gene encoding PCFT is located on chromosome 17q11.2. The major physiological role of PCFT is absorption of dietary folates through upper gastrointestinal tract where it functions optimally at acidic pH-5.5. It functions by coupling the flow of protons down an electrochemical concentration gradient to the uptake of folates into cells.¹¹³ Its activity at the low pH and the structural specificity (high affinity for folic acid, and low affinity for PT523, a non-polyglutamable analog of aminopterin) distinguishes itself functionally from
the RFC (optimal activity at pH 7.4, very low affinity for folic acid and very high affinity for PT523).

2.3.1. Structure of PCFT

![Figure 19. Structure of PCFT (modified from ref.113).](image)

hPCFT consists of 459 amino acids with a molecular mass of 49.8 kDa. It is predicted to include 12 TMDs with N- and C-termini towards cytoplasm (Figure 19). The loop domain between the first and second TMDs must be extracellular because the two putative N-glycosylation consensus sites in this region are glycosylated. N-glycosylation does not appear to be required for either PCFT trafficking or function.113
2.3.2. PCFT expression patterns in human tissue

PCFT is expressed in the apical membrane of the proximal jejunum and in many normal tissues including retina, brain liver, kidney and placenta.\textsuperscript{115} The highest PCFT levels within the intestine are found in the proximal jejunum and duodenum. Here, it serves as the primary means of intestinal uptake of dietary folates at the acidic pH characterizing the upper small intestine.\textsuperscript{116}

2.3.3. Transport mechanism of PCFT

The uphill flow of folates into cells is coupled with the downhill flow of protons \textit{via} PCFT, and so, PCFT functions as a folate-proton symporter. Consistent with a proton-coupled process, a transvesicular pH gradient results in an increased unidirectional transport of folate and substantial transmembrane folate concentration gradients from the low-pH to the high-pH compartment. One of the unique characteristics of PCFT involves its acidic pH optimum i.e. transport is maximal at pH 5-5.5, but as the pH increases above 5.5, transport decreases significantly and above pH 7, there is no activity.\textsuperscript{117}

2.3.4. PCFT and antifolate chemotherapy

The interstitial pH of solid tumors favors PCFT transport as it is acidic. A prominent low-pH transport route was identified in a substantial cohort of solid human tumors, and high levels of hPCFT transcripts were reported in a broad range of human tumors.\textsuperscript{118} Whereas the role of tumor selectivity and PCFT in antifolate activity is still evolving, transport of classical antifolates by PCFT has been described previously,\textsuperscript{117} although
classical antifolate PMX shows high affinity for the carrier at both acidic and neutral pHs. PMX is transported by both PCFT as well as RFC, and so its transport into normal tissues via RFC could be the major reason behind its lack of selectivity for tumor cells over normal cells. Thus, this provides a strong rationale for designing and developing folate-based therapeutics that are substrates for transporters with limited transport in normal cells compared with tumors. Thus, both FRs and PCFT can be used for targeted cancer therapy.\textsuperscript{86,120}
3. Glycinamide-ribonucleotide formyltransferase (GARFTase)

Figure 20. Proposed mechanism for GARFTase catalysis of GAR to Fgar.\textsuperscript{121}

GARFTase, involved in purine synthesis, is one of the most important trifunctional enzymes.\textsuperscript{122} As mentioned above, it catalyzes the transfer of the formyl group from $N^{10}$-CHO-THF to the primary side-chain amino group of GAR to yield FGAR and THF (Figure
20). It ultimately incorporates C-8 into IMP. Inhibition of *de novo* purine biosynthesis via GARFTase inhibition is a viable approach for cancer chemotherapy.\(^{86,123-124}\)

### 3.1. Structure of GARFTase

The structure of hGARFTase has been reported at pH 4.2 (1.7 Å), pH 8.5 (2 Å), pH 8.5 in the binary complex with the substrate β-GAR (2.2 Å) and at pH 7 in a binary complex with the co-substrate analog inhibitor 10-trifluoroacetyl-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic acid (10-CF\(_3\)CO-DDACTHF) (2 Å)\(^{125}\) and folate inhibitors.\(^{126}\) Crystal structure of hGARFTase with pemetrexed and 6-substituted pyrrolo[2,3-\(d\)]pyrimidine antifolate with β-GAR substrate was reported recently.\(^{120}\)

### 3.2. Catalytic Mechanism of GARFTase

The formyl group is transferred by a nucleophilic attack of the GAR amino group on the formyl carbon of the co-substrate leading to the formation of a tetrahedral intermediate. Kinetic studies of the *E. coli* enzyme\(^{127}\) and of the human enzyme\(^{128}\) and murine enzyme\(^{129}\) GARFTase domains suggested this mechanism. It is proposed that a “fixed” water molecule mediates the required proton transfer between substrate and cofactor, though, it has not been verified experimentally.\(^{130}\) A proposed mechanism for GARFTase is shown in Figure 20. The cofactor binds to the active site, and Asp144 forms a salt bridge with the imidazolium of His108. The formyl group is positioned to form hydrogen bonds with the protonated imidazolium group of His108 and Asn106. Tetrahedral intermediate is formed when the free base form of the amino group of GAR
attacks the activated formyl group. Catalytic water molecule mediates a proton transfer from GAR to the $N^{10}$ of folate, followed by breakdown of the tetrahedral intermediate to form products. The positioning of this water molecule may be assisted by a hydrogen bond to the carboxylate of Asp144.\textsuperscript{121}

3.3. Binding of Inhibitors

Zhang et al.\textsuperscript{125} reported a 1.98 Å crystal structure of hGARFTase in a binary complex with the co-substrate analog inhibitor 10-CF$_3$CO-DDACTHF (Table 2) at pH 7 (PDB ID = 1NJS). The cofactor binding pocket of GARFTase is located at the interface between the the C-terminal of the structure and the N-terminal mononucleotide binding domain. The binding site for the folate cofactor moiety consists of three parts: the pteridine binding cleft, the benzoylglutamate region, and the formyl transfer region.

\textbf{Figure 21.} Crystal structure of hGARFTase. (PDB:4ZZ1 and PDB:4ZZ2)\textsuperscript{120}
Gangjee and coworkers\textsuperscript{120} published the crystal structure of hGARFTase with pemetrexed and 6-substituted pyrrolo[2,3-\textit{d}]pyrimidine antifolate (Figure 21) and $\beta$-GAR substrate recently. According to the crystallographic models, antifolates form six hydrogen bonds via the pyrrolo[2,3-\textit{d}]pyrimidine moiety with hGARFTase peptide backbone. Moreover, two hydrogen bond interactions with the peptide backbone, a bidentate polar interaction with Arg871, and three charge–charge interactions with Arg897 and Lys844 are mediated \textit{via} the glutamate tail of the antifolates.\textsuperscript{120}

**Microtubules - Summary**

Microtubules are important for various functions like transcription, translation, permeability, centrosome clustering etc. in interphase cells. Microtubules is also important for trafficking of protein and nucleic acid cargo in cancer cells, and so MTAs are highly successful anticancer agents. These drugs disrupt microtubule dynamics leading to microtubule polymerization or depolymerization, and thus classified as microtubule stabilizing agents like Taxanes, and destabilizing agents like Vinca alkaloids or colchicine site agents. The problem with current agents is multidrug resistance caused by structural alteration of the microtubules and drug efflux by ATP binding cassette (ABC) proteins. The MTAs can be combined with antiangiogenic agents. Agiogenesis is the process of formation of new blood vessels from existing vascular beds. Release of the pro-angiogenic growth factors lead to their binding with transmembrane receptors of the protein kinase family identified as RTKs, namely, vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor
(PGFR). These initiates signal transduction and angiogenesis.

Tumor vessels are often dilated, and have a haphazard distribution of vessels. The heterogeneity of tumor vasculature can be caused due to the excessive vessel leakiness leading to escape of plasma. This heterogeneity leads to hypovascular and hypervascular areas in tumor with sluggish blood flow in some regions and excessive in others. The heterogeneous blood perfusion occurs spatially as well as temporally. These static regions also lead to the hypoxic conditions in the cell as well as low extracellular pH. Due to this, the administered cytotoxic drugs cannot penetrate well in these static regions of a tumor cell. According to vascular normalization hypothesis formulated by Jain and coworkers, the antiangiogenic agent transiently normalizes this vasculature by pruning the abnormal vessels and remodeling the remaining ones which leads to reduction in hypoxia and interstitial fluid pressure. As a result, there is improved delivery of the cytotoxic agent to the tumor thereby explaining, in part, the synergy observed in the use of antiangiogenic and cytotoxic drug combinations. Using multitargeted agents also increase therapeutic effectiveness, preventing cancer cells from developing resistance, which is the principal cause of failure of current chemotherapy. Other advantages include a lower risk of drug-drug interactions in vivo and improved patient compliance resulting from reduction in the number of medications required. At the level of biochemical response, a single agent with multiple targets would be less prone to interact with anti-targets compared to multiple separate agents which would be more prone to produce off-target toxicities. So, a single agent would have less adverse effects, if any, compared to multiple agents.
Folates- Summary

The lack of tumor selectivity for all the currently clinically used antifolates reflects their cellular uptake by the reduced folate carrier (RFC) expressed ubiquitously by both normal tissues as well as tumors. In contrast, folate receptor \((\text{FR})\) \(\alpha\) and \(\beta\), and the proton-coupled folate transporter (PCFT) exhibit selective tumor tissue expression, and serve specialized physiological roles.\(^{113}\) FRs on normal cells are inaccessible to circulating folates or are non-functional in normal tissues. PCFT is expressed in the upper gastrointestinal tract where it functions only at acidic pH as the major intestinal transporter for absorption of dietary folates.\(^{131}\) Given this requirement of acidic pH for activity, PCFT transport is limited in normal tissues that are not at acidic pH (<6.9). A substantial cohort of solid tumors (eg. ovarian, non-small cell lung cancer) express PCFT, often in concert with FRs. FR\(\alpha\) is accessible on tumors, thus, allowing tumor targeting by this mechanism. FR\(\beta\) is expressed in hematological malignancies and in white blood cells of the myeloid lineage, including tumor-associated macrophages (TAMs). FR\(\beta\)-positive TAMs may play an important role in the tumor microenvironment in reaction to tumor metastasis and angiogenesis by releasing proangiogenic factors suggesting that TAMs may constitute an additional potential therapeutic target in cancer for FR\(\beta\)-targeted agents.\(^{132}\) There are no tumor targeted cytotoxic agents in clinical use, thus, all cytotoxic agents display dose-limiting toxicity. New cytotoxic agents are needed which can target tumor cells selectively by virtue of their specificities for FRs and /or PCFT and thus, afford agents that do not suffer from dose-limiting toxicities.\(^{133}\)
II. CHEMICAL REVIEW

The chemistry related to the work described in this dissertation is reviewed in this section.

1. Iodination

2. Cyanation

3. Trifluoromethylation

4. Pyrrolo[2,3-\textit{d}]pyrimidines ring system formation

1. Iodination

   • Using sodium iodide

   Scheme 1. Exchange of chlorides with iodides using sodium iodide and trimethylsilyl chloride.

   \[
   \begin{align*}
   \text{11} & \quad \text{Cl} \quad \text{N} \quad \text{N} \quad \text{I} \quad \text{N} \quad \text{N} \quad \text{CF}_3 \\
   & \quad \text{CO}_2\text{Et} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{CF}_3 \\
   \end{align*}
   \]

   Reactions and conditions: a) NaI, TMS-Cl, MeCN, 180 °C, MW
Yang et al.\textsuperscript{134} and Lemoine et al.\textsuperscript{135} exchanged the chlorides in 11 and 13 respectively with iodides with NaI and TMS-Cl in acetonitrile at 180 °C in a microwave reactor (Scheme 1).

- **Using iodo(trimethyl)silane**

  **Scheme 2.** Exchange of chlorides with iodides using iodo(trimethyl)silane.

  ![Chemical structure](image)

  Reactions and conditions: iodo(trimethyl)silane, MeCN, 81 °C, 4 h, 79%

  Zehnder et al.\textsuperscript{136} added iodo(trimethyl)silane to the suspension of 15 in MeCN to get to the 2-amino-4-iodo-5,7-dihydro-6H-pyrrolo[3,4-d]pyrimidine 16 (Scheme 2).

- **Using hydroiodic acid**

  **Scheme 3.** Exchange of chlorides with iodides using aqueous hydroiodic acid.

  ![Chemical structure](image)
Reactions and conditions: aq. HI, MeCN, 0 °C- rt

Hughes et al.\textsuperscript{137} treated 4-chloro-1-methyl-6,8-dinitropyrrorolo[4,3,2-de]quinolin-2(1H)-one 17 with hydroiodic acid to yield iodo 18 (Scheme 3). Yields were not mentioned.

2. \textit{Cyanation}

- Using copper cyanide

\textbf{Scheme 4.} Formation of 3-nitropicolinonitrile

\text{Reactions and conditions: CuCN, fusion 150 °C, 2 h, 68%}

Loidreau et al.\textsuperscript{138} reacted 2-bromo-3-nitropyridine 19 with copper cyanide to 3-nitropicolinonitrile 20 (Scheme 4).

\textbf{Scheme 5.} Transformation of iodide into the nitrile moiety using copper cyanide.
Reactions and conditions: CuCN, pyr, 80 °C

Hughes et al.\textsuperscript{137} transformed N,N'-\(4\text{-}\textit{iodo-1\text{-}methyl-2\text{-}oxo-1,2\text{-}dihydropyrrolo[4,3,2-de]quinoline-6,8\text{-}diyl}\)bis(2,2,2-trifluoroacetamide) \textbf{21} into the nitrile \textbf{22} employing copper cyanide in pyridine (Scheme 5).

3. \textbf{Trifluoromethylation}

Wang et al.\textsuperscript{139} used a novel strategy for aromatic trifluoromethylation by converting aromatic amino group into a trifluoromethyl group. It was the trifluoromethylation variation of the classic Sandmeyer reaction.

\textbf{Scheme 6}. Treatment of iodide with copper and diphenyl-(trifluoromethyl)sulfonium trifluoromethanesulfonate

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=0.4\textwidth]{example.png}};
\end{tikzpicture}
\end{center}

Reactions and conditions: CF\textsubscript{3}SO\textsubscript{3}+S-(CF\textsubscript{3})(Ph)\textsubscript{2}, Cu, DMF, 60 °C, 18 h, 80%

Georgsson et al.\textsuperscript{140} generated the trifluoromethyl-substituted pyrrolo[2,3-\textit{c}]pyridine \textbf{24} by treating ethyl 5-\textit{iodo-H-pyrrolo[2,3-c]pyridine-2-carboxylate} \textbf{23} with copper and diphenyl-(trifluoromethyl)sulfonium trifluoromethanesulfonate in DMF (Scheme 6).
Stout et al.\textsuperscript{141} attempted trifluoromethylation of 8a-hydroxy-8-methyl-octahydroimidazo-
cyclopenta[1,2-e]pyrrolo[1,2-a]pyrazinedione 25 following the protocol of Baran and co-
workers\textsuperscript{142} (tBuOOH, aqueous NaSO$_2$CF$_3$, or ZnSO$_2$CHF$_2$, respectively) which smoothly
converted the starting material to the trifluorinated analogue 26.

\textbf{Scheme 7. Trifluoromethylation using NaSO$_2$CF$_3$}

![Scheme 7](image)

Reactions and conditions: NaSO$_2$CF$_3$, tBuOOH, H$_2$O, rt, 46 h, 49%  

\textbf{4. Synthesis of 5-substituted pyrrolo[2,3-d]pyrimidines}

The synthetic procedures for substituted pyrrolo[2,3-d]pyrimidines are already well
established as published by Gangjee and coworkers.

\textbf{a. From Pyrroles}

Pyrimidine is the primary precursor in the literature for the synthesis of pyrrolo[2,3-
d]pyrimidines (Figure 22) and the formation of bonds between atom 4a, 5 and atom 6 and 7 is described in the literature.\textsuperscript{143-144}

Reactions and conditions: a. DMF, 60 °C, 48 h, 20-45%

Secrist and Liu$^{145}$ reacted 2,6-diamino-4-hydroxypyrimidine 27 with various α-halo aldehydes and ketones (Scheme 8). Cyclization occurred via two modes to produce either
the pyrrolo[2,3-\textit{d}]pyrimidine and/or the furo[2,3-\textit{d}]pyrimidine. Thus α-halo ketones, chloroacetonitrile 28 and 3-bromo-2-butanone 29, yielded both the furo[2,3-\textit{d}]pyrimidine 30 and the pyrrolo[2,3-\textit{d}]pyrimidine 31, whereas 32 produced only the pyrrolo[2,3-\textit{d}]pyrimidine 34 on reaction with 33.

\textbf{Scheme 9}. Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines 38.

Reactions and conditions:  
a. guanidine HCl, HCl solution  
b. heat, 120 °C, 5 h

Seela and Luepke\textsuperscript{143} reported 6-substituted 2-amino-3,7-dihydropyrrolo[2,3-\textit{d}]pyrimidin-4-one 38 synthesis from 1,3-dioxolane-2-propanoic acid, α-cyano-2-methyl-ethyl ester 35 and guanidine 36 (Scheme 9).
Noell and Robins\textsuperscript{146} reported the synthesis of unsubstituted pyrrolo[2,3-d]pyrimidines 41 (Scheme 10) and 43 by the reaction of chloroacetaldehyde 40 with 2-amino-6-alkylamino-4-hydroxypyrimidines 39 and 6-amino-1,3-dimethyluracil, 42 respectively.

**Scheme 10.** Synthesis of pyrrolo[2,3-d]pyrimidines 41 and 43

Reactions and conditions: 55-60 °C, 30 min, 35-89%

Scheme 11. 5-substituted, and 5,6-disubstituted pyrrolo[2,3-d]pyrimidine.
Reactions and conditions:  

a) NaOAc, H$_2$O, 50 °C, 20 h, 83%

b) NBS, DMF, rt, 6 h, 88%

Gibson et al.\textsuperscript{147} reported the synthesis of 5,6-disubstituted-pyrrolo[2,3-d]pyrimidine. 2-Chloro-2-cyanoethanal 45 (Scheme 11) was reacted with 44 to furnish 7-yano-7-deazaguanine 46. Substituents at C-5 and C-6 were obtained by brominating deazaguanine 46 with $N$-bromosuccinimide in DMF to yield 7-cyano-8-bromo derivative 47.

Taylor et al.\textsuperscript{148} reported synthesis of PMX (Scheme 12) involving cyclization of sodium 4-[3-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-4-nitrobutyl]benzoate 49. Acid 50 was converted to PMX and involved peptide coupling with diethyl $L$-glutamate using 2-chloro-4,6-dimethoxy-1,3,5-triazine as the coupling agent in presence of $N$-methylmorpholine and final saponification.

\textbf{Scheme 12.} Synthesis of pyrrolo[2,3-d]pyrimidine 50 from 6-amino-5-pyrimidylacetaldehydes.

Reactions and conditions: a. i) aq. NaOH, rt, 2 h ii) HOAc, overall 57%

b. i) L-Glu.HCl, NMM, CDMT, rt, 62% ii) I N NaOH, H$_2$O, rt, 73%

51

Wright, G. E. *et al.*\textsuperscript{149} reported acid-catalyzed (Fisher indole) cyclization of 6-(phenylhydrazino)uracils 51 (Scheme 13) to 9H-pyrimido[4,5-b]indole-2,4-diones 52. Several authors\textsuperscript{150-154} have employed the Fischer indole cyclization of 4-pyrimidinylhydrazones 53 (Scheme 13) to afford the pyrrolo[2,3-d]pyrimidine ring system 54.

Reactions and conditions: a. Pd(OAc)\(_2\), b. NaN\(_3\), EtOH, reflux, 12 h; c. 180 °C, 10 h, 62-90%

Kondo and coworkers\(^{155}\) synthesized pyrrolo[2,3-\(d\)]pyrimidine 60 (Scheme 14) via thermal cyclization of 4-azidopyrimidines 59 containing an olefinic moiety at the 5-position. Intermediates 58 were obtained by a palladium catalyzed cross-coupling between the 5-iodopyrimidine 55 and appropriate stannanes 56 or 57, followed by nucleophilic displacement of the 4-chloro in pyrimidine 58 with sodium azide. Yields were only mentioned for the final step.

Gangjee and co-workers\(^{156}\) reported the synthesis of 2-amino-4-methylpyrrolo[2,3-\(d\)]pyrimidine (Scheme 15). Compound 61 and guanidine carbonate were refluxed with absolute ethanol in the presence of triethylamine or sodium methoxide to afford 62 which was converted to 63 under reflux conditions with phosphorus oxychloride. Condensation
of benzylamine with 63 in the presence of triethylamine under reflux in n-BuOH afforded the bicyclic compound 64. Compound 64 was oxidized to aromatic compound 65 using MnO₂. Sodium in liquid ammonium at -78 °C afforded the debenzylation of 65 to 66.

**Scheme 15.** Synthesis of pyrrolo[2,3-\textit{d}]pyrimidine 66.

Reactions and conditions: a. guanidine carbonate, EtOH, Et₃N, reflux, 1 h, 69%; b. POCl₃, reflux, 2 h, 68%; c. benzylamine, Et₃N, n-BuOH, 90 °C, 3 days, 50%; d. MnO₂, dioxane, reflux, 24 h, 45% e. Na/liq. NH₃, -78 °C, 65%.

Legraverend and coworkers\textsuperscript{157} reported the synthesis of pyrrolo[2,3-\textit{d}]pyrimidines 71 (Scheme 16) from 2-amino-4,6-dichloro-5-(2,2-diethoxyethyl)pyrimidine 68. 5-allyl-2-
amino-4,6-dihydroxypyrimidine was prepared by the reaction of guanidine hydrochloride with diethyl allylmalonate, and then converted to the 4,6-dichloro derivative by treatment with POCl₃. The (2-amino-4,6-dichloropyrimidin-5-yl)acetaldehyde 67 was obtained by ozonolysis of the allyl group. The acetal 68 was cyclized to 2-amino-4-chloro-7-alkyl-7H-pyrrolo-[2,3-d]pyrimidine 70 by treatment with dilute aqueous HCl at room temperature. Compound 71 was then obtained by hydrolysis of the 4-chloro group of 70 using 1N HCl at 100 °C.


Reactions and conditions: a. Et₃N, BuOH, 100 °C, 2 days, 42%; b. 0.2N HCl, rt, 3 days, 69%; c. 1N HCl, 100 °C, 6 h, 85%
**Scheme 17.** Synthesis of pyrrolo[2,3-\textit{d}]pyrimidines 74.

![Scheme 17](image)

Reactions and conditions:  
a. Et₄NCl, Pd(PPh₃)₂Cl₂, MeCN, reflux, 2 h, 11-71%; b. Conc. HCl, MeOH, reflux, 20-40 min, 71-96%.

Sakamoto et al.¹⁵⁸ synthesized pyrrolo[2,3-\textit{d}]pyrimidines 74 (Scheme 17) by intramolecular cyclization of protected 5-acetaldehyde pyrimidines 73. Compounds 73 were in turn synthesized by palladium(0) catalyzed coupling of the appropriate 2,4-disubstituted-5-bromo-6-acetamido pyrimidines 72 with (Z)-1-ethoxy-2-(tributylstannyl)ethane 75.

**b. From Pyrroles**

Taylor et al.¹⁴⁴ reported the synthesis of 4-amino-5-carboxylic acid-pyrrolo[2,3-\textit{d}]pyrimidine 78 (Scheme 18). Tetracyanoethylene 75 was converted by the action of hydrogen sulfide to 2,5-diamino-3,4-dicyanothiophene 76, which was rearranged to 5-amino-3,4-dicyano-2-mercaptopyrrole 77 as described by Middleton, et al.¹⁵⁹
of 77 with methyl orthoformate followed by alcoholic ammonia afforded 4- amino-5-cyano-6-methylmercaptopyrrolo[2,3-\(d\)]pyrimidines 78. Raney nickel desulfurization of 78 then yielded 79. Exact yields and reaction conditions were not provided for the intermediates.

**Scheme 18.** Synthesis of 4-amino-5-carboxylic acid-pyrrolo[2,3-\(d\)]pyrimidine 79.

Reactions and conditions:  a. H\(_2\)S; b. Alkali c. Orthoformate, NH\(_3\) in ethanol d. 1N NaOH, 35%.

In another report, Taylor et al.\(^{160}\) synthesized 4-amino-5-methyl-pyrrolo[2,3-\(d\)]pyrimidine 84 (R = CH\(_3\)) (Scheme 19) from 2-amino-3-cyano-5-substituted pyrroles 82 which in turn were obtained from malononitrile and the appropriate \(\alpha\)-aminoketones 80. Treatment of pyrrole 82 with triethylorthofomrate followed by ammonia resulted in the imidene intermediates 83 which underwent cyclization with sodium methoxide in pyridine to afford
the pyrrolo[2,3-\(d\)]pyrimidine 84.

**Scheme 19.** Synthesis of 4-amino-5-substituted pyrrolo[2,3-\(d\)]pyrimidines 84.

```
R = CH₃, C₆H₅
```

Reactions and conditions:  a. malonitrile, EtOH, reflux, 67%; b. i. triethyl orthoformate, reflux, 5 h, ii. NH₃; c. i. triethyl orthoformate, reflux, 5 h, ii. NH₃, 34-78%; d. NaOMe, pyridine, reflux, 1 h, 91%

Taylor and coworkers\textsuperscript{161} reported the synthesis of a 5,6-dihydro-pyrrolo[2,3-\(d\)]pyrimidine analog of PMX by a novel route (Scheme 20). A manganic triacetate dehydrate-mediated radical cyclization of racemic methyl \(N\)-crotyl-\(N\)-[1-(3,4-phenyl)-ethyl-1-yl]malonamide 89, afforded a diastereomeric mixture of the 3-carboxethoxy-2-pyrrolidinone 90.\textsuperscript{162} Compound 87 was in turn obtained by alkylation of racemic 1-(3,4-dimethoxy-phenyl)-ethylamine 84 with crotyl bromide 86 followed by a DMAP catalyzed acylation with methyl malonyl chloride 88. The pyrrolidinone 90 was converted to the
thiolactam 91 with $P_2S_5$ followed by cyclocondensation with guanidine to afford the $N^7$-protected 5,6-dihydro-5-allyl-pyrrolo[2,3-$d$]pyrimidine 92. Palladium catalyzed cross coupling with diethyl-4-iodobenzoyl-$L$-glutamate fortuitously afforded the ethano-bridged derivatives, and not the expected vinyl-bridged derivatives, by double bond migration. This compound was then elaborated to analogs of PMX.

**Scheme 20.** Synthesis of $N^7$-substituted analogs of PMX.

Reactions and conditions:  

a. NaH, TBAI, THF, 0 °C-rt, 10 h, 82%; b. DMAP, Et$_3$N, EtOAc, 0 °C-rt, 2 h, 88%; c. Mn(OAc)$_3$, H$_2$O, Cu(OAc)$_2$, Acetic acid, rt, 18 h, 97%; d. $P_2S_5$, THF, reflux, 3 h, 68%; e. guanidine HCl, MeOH, rt, 30 min, 50%.

Barnett *et al.*$^{163}$ reported the synthesis of a 2-amino-4-oxo-5,6-dihydropyrrolo[2,3-$d$]pyrimidine 98 (Scheme 21) *via* a guanidine cyclization of a preformed 3-carbethoxy-2-
thiopyrrolidine intermediate 97 as the key step. This intermediate was in turn prepared in several steps from 4-propionaldehyde benzoic acid tert-butyl ester 93. Compound 98 was reduced to the pyrrolo[2,3-\(d\)]pyrimidine intermediate 99, which was then elaborated to PMX in several steps. Reaction conditions are not provided in the publication.

**Scheme 21.** Synthesis of pyrrolo[2,3-\(d\)]pyrimidine 99 and PMX.

Reactions and conditions: a. diethyl malonate, TiCL\(_4\)/Py, THF; b. MeNO\(_2\), DBU, 72% over 2 steps; c. H\(_2\), Pd/C, 42%; d. P\(_2\)S\(_5\), 77%; e. TFA, guanidine, 64%; f. L-Glu, NMM, CDMT, DMF, 52%; g. IN NaOH, 92%
Girgis, Jørgensen, and Pedersen\textsuperscript{164} reported the synthesis of 2-methyl-pyrrolo[2,3-d]pyrimidines 101 (Scheme 22). Compounds 101 were obtained in a one-step cyclization of the corresponding 2-acylamino-3-cyanopyrrole derivatives 100. The reaction was carried out by heating compound 100 in a mixture of phosphorus pentaoxide, N-N-dimethylcyclohexanamine, and water. It is believed that the formation of 101 proceeds through the intermediacy of the amides 102 resulting from hydrolysis of the cyano function in 100 followed by intramolecular cyclodehydration under reaction condition. Yields were not mentioned in the publication for the intermediates.

**Scheme 22. Synthesis of 7-deazahypoxanthines 101.**

Reactions and conditions: a. 85\% H\textsubscript{3}PO\textsubscript{4}. 130 \degree C; b. 50 \degree C, 3-5 min c P\textsubscript{2}O\textsubscript{5}/DMCA/H\textsubscript{2}O. 180 \degree C, 2 h, 43\%
Chen et al. \(^{165}\) reported an efficient synthesis of pyrrolo[2,3-\(d\)]pyrimidine 112 (Scheme 23). Knoevenagel condensation of malononitrile 103 and excess acetone in refluxing toluene or benzene gave 105. Bromination of 105 using NBS and benzoyl peroxide in chloroform afforded 106 as a major component contaminated with undesired dibromo derivative 107 and starting material which were separated using column chromatography. Cyclization of 106 with aryl amine 110 afforded the substituted pyrrole intermediate 111. Compound 111 was then cyclized to the pyrrolo[2,3-\(d\)]pyrimidine 112 using 85% phosphoric acid.

**Scheme 23.** Synthesis of 2,5,-dimethyl-\(N^7\)-substitutedpyrrolo[2,3-\(d\)]pyrimidine 112.

Reactions and conditions: a. NaOAc, AcOH, benzene, reflux, 4 h, 70.1%  
b. NBS, benzoyl peroxide, CHCl\(_3\), reflux, 15 h, 77%;  
c. rt, 15 h, 60%;  
d. 85% phosphoric acid, 130 °C, 30 min, 81.1%

c. **From Furans**

**Scheme 24.** Synthesis of 2,5,6-trisubstituted pyrrolo[2,3-\(d\)]pyrimidines 115.
Reactions and conditions: NaSO$_2$CF$_3$, tBuOOH, H$_2$O, rt, 46 h

Taylor and coworkers$^{166}$ (Scheme 24) reported that 2-amino-3-cyanofurans 113 on cyclization with amidines 114 afforded the corresponding pyrrolo[2,3-$d$]pyrimidines 115 by an unexpected ring transformation/ring annulation sequence.

Several plausible mechanisms for the above amidine-mediated ring transformation during annulation reactions have been proposed. Michael addition of the amidine to the 2-position of the furan ortho-aminonitrile 113 (Scheme 25) followed by furan ring cleavage generates an open-chain carbonyl derivative 116, which recyclizes to pyrrole, thus incorporating the 2-amino group of the furan aminonitrile starting material as the pyrrole NH. Subsequent intramolecular addition of the amidino substituent to the ortho-substituted nitrile group then completes the pyrimidene ring annulation to afford 115. Alternatively, deprotonation of the acidic 2-amino group of the furan O-aminonitrile 116 by the strongly basic amidine could be followed by C-O bond cleavage to generate an intermediate ketenimine which would lead to the substituted malononitrile 116. Further reaction with the amidine in the usual way would lead to a 2-substituted-4,6-diaminopyrimidine carrying a $\beta$-carbonyl substituent at C-5, and fused pyrrole ring would then be formed with a final ring closure.
Scheme 25. Plausible mechanisms for amidine-mediated ring transformation

Various iodination reactions were reviewed in this section and electrophilic iodinating agents did not work well with pyrimidine systems, and the reason could be that the pyrimidine ring is an electron poor system. Various nucleophilic iodinating agents like
sodium iodide, iodonitrimethylsilane, hydroiodic acid have also been reviewed. 57% Hydroiodic acid in water worked successfully. One of the reasons might be the lower temperature (0 °C – rt) this reaction was attempted at. Other nucleophilic reactions were high temperature reactions at 80-180 °C. For the synthesis of pyrrolo[2,3-\textit{d}]pyrimidines, the biggest concern was the formation of side product furo[2,3-\textit{d}]pyrimidine. Solvent plays a major role for these kinds of reactions. For e.g., it was found that with DMF, both products pyrrolo[2,3-\textit{d}]pyrimidine, and furo[2,3-\textit{d}]pyrimidine are formed but with methanol-water mixture, only former product pyrrolo[2,3-\textit{d}]pyrimidine is formed. Although many methods were tried as mentioned in the chemical review section, it was decided that using 2, 6-diamino-4-hydroxypurimidines as starting material, it will not lead to any production of 6-substituted pyrrolo[2,3-\textit{d}]pyrimidine with bromo-aldehyde intermediates. Taylor\textsuperscript{145} used the similar method for the synthesis of PMX as mentioned earlier. Fischer Indole cyclization has been specifically employed for the synthesis of 6-substituted or 5,6 substituted products. Gangjee and co-workers\textsuperscript{156} synthesized 2-amino-4-methylpyrrolo[2,3-\textit{d}]pyrimidine with guanidine carbonate as starting material. The method works very well for synthesis of N-benzylated products with 4-methyl. Although synthesis of pyrrolo[2,3-\textit{d}]pyrimidine is less popular, various reactions were reviewed and described earlier in the chemical review section. Taylor also synthesized pyrrolo[2,3-\textit{d}]pyrimidine from 2-amino-3-cyano-5-substituted pyrroles. This reaction led to the formation of 5-substituted product but without any substitution at 2-position. Same product was also formed from tetracyanoethylene which led to formation of 2,5-diamino-3,4-dicyanothiophene which was rearranged to 5-amino-3,4-dicyano-2-mercaptopyrrole.
These reactions used basic conditions as well but took two more steps for the formation of the product.
III. STATEMENT OF THE PROBLEM

Antiangiogenic agents

Beyond 2 mm$^3$, a tumor requires additional oxygen and nutrients for maintenance and growth, and initiates the process of developing a blood supply system. Under hypoxic conditions, several proangiogenic factors are secreted, of which VEGF is the key mediator of angiogenesis. VEGF binds to its receptor VEGFR2, and stimulates the process of angiogenesis which supports rapid tumor growth and metastases. Developing VEGFR2 kinase inhibitors is one of the key strategies to obtain antiangiogenic effects. These inhibitors arrest tumor growth, and cause tumor shrinkage. However, resistance to VEGFR2 inhibitors results in increased invasion and metastases. At the onset of VEGFR2 inhibition there are two effects:

1. Regression of tumor vessels
2. Transient normalization of surviving vessels.

Normalization

Tumor vessels are often dilated, and have a haphazard distribution of vessels. The heterogeneity of tumor vasculature can be caused due to the excessive vessel leakiness leading to escape of plasma. This heterogeneity leads to hypovascular and hypervascular areas in tumor with sluggish blood flow in some regions and excessive in others. The heterogeneous blood perfusion occurs spatially as well as temporally. These static regions also lead to the hypoxic conditions in the cell as well as low extracellular pH. Due to this,
the administered drugs cannot penetrate well in these static regions of a tumor cell.\textsuperscript{73} According to vascular normalization hypothesis formulated by Jain and coworkers, the antiangiogenic agent transiently normalizes this vasculature by pruning the abnormal vessels and remodeling the remaining ones which leads to reduction in hypoxia and interstitial fluid pressure. The treatment with bevacizumab (anti-VEGF antibody) and DC101 (anti-VEGFR2 antibody) reduced the size and length as well as permeability of these abnormally dilated and tortuous vasculature in human colon cancer and glioma xenografts as well as murine breast cancers so that it more closely resembled the normal vasculature. Bevacizumab also reduced the intratumoral microvessel density (MVD), but not the density of mature vessels which are pericyte-covered MVD). This indicates that during normalization, only immature vessels, which lack pericytes are pruned, and not mature vessels.\textsuperscript{171} Therefore, the ‘normalized’ vasculature with bevacizumab treatment has greater perivascular cells coverage with thicker and normal vasculature resembling basement membrane in breast cancers, squamous cell carcinomas and gliomas. It also leads to the normalization of tumor microenvironment characterized by reduced interstitial fluid pressure due to the decrease in the vessel leakiness. Pressure gradient is also restored across wall of the blood vessels as well as tumor interstitium. Overall antiangiogenic agents lead to increased drug penetration in tumors.

Investigations into the combined use of VEGFR2 inhibitors with cytotoxic therapies have yielded more promising results than VEGF-targeting monotherapy.\textsuperscript{71, 172} Combinations of cytotoxic agents and VEGFR2 inhibitors have shown synergistic effects
suggesting that there is improved delivery of the cytotoxic agent during this transient phase of vessel normalization.\textsuperscript{172}

Yoshizawa \textit{et al.}\textsuperscript{76} reported that paclitaxel (cytotoxic) in combination with the VEGFR2 inhibitor semaxanib (cytotoxic) enhanced tumor penetration and in vivo antitumor activity in Colon-26 solid tumor-bearing mice. Semaxanib treatment increased the distribution of paclitaxel in the core region of the tumor, and hence, decreased (the ratio of) its peripheral distribution. Thus, utilizing VEGFR2 inhibition-based vascular normalization afforded transient enhanced blood supply to the tumor and allowed the delivery of the cytotoxic agent.\textsuperscript{76}

\textbf{Scheduling of the cytotoxic agent}

The effects of the antiangiogenic agent on the tumor and the intratumoral levels of the cytotoxic agent affect the outcome of combination chemotherapy.\textsuperscript{173} To maximize the intratumoral levels of the cytotoxic agent, administration of the cytotoxic agent needs to be appropriately scheduled to ensure that it is used during the period of transient vessel normalization by the antiangiogenic agent.\textsuperscript{174} Tumor-targeted drug delivery has the potential to improve cancer treatment by reducing off-target toxicities and increasing the efficacy of the cytotoxic agent.\textsuperscript{175} Judicious scheduling is an influential and critical factor because of the impact on intratumoral cytotoxic drug levels.\textsuperscript{169, 174, 176, 177} Hence, if not appropriately scheduled, the cytotoxic agent may miss the timing window of transient normalization.\textsuperscript{178} Also, administering separate antiangiogenic and cytotoxic agents may
not alleviate the issues of dose limiting toxicities of cytotoxic agents that are not tumor selective.

Compounds with antiangiogenic and cytotoxic activities in single agents

Single agents with both antiangiogenic and cytotoxic activities have several advantages:

1. Antiangiogenic and cytotoxic activities in a single agent would allow the cytotoxicity to be manifested as soon as the antiangiogenic effects are operable. These multitargeted agents could exert their cytotoxic action as soon as, or even during transient tumor vasculature normalization due to the antiangiogenic effects. Several literature reports indicate the success of using antiangiogenic agents with metronomic doses of cytotoxic agents.\(^\text{66-69, 179}\)

2. These multitargeted single agents would circumvent the pharmacokinetic problems of two or more agents dosed separately, would avoid drug-drug interactions at receptor as well as chemical level, could be devoid of overlapping toxicities, and could delay or prevent tumor cell resistance.\(^\text{180}\)

3. Reduced cost and increased patient compliance are some of the other advantages of such single agents which are sometimes as significant contributors to chemotherapy failure as resistance, toxicity, and lack of efficacy.\(^\text{180}\)

4. Dose-limiting toxicity is one of the major issues with conventional cytotoxic chemotherapeutic agents. These single agents should avoid these toxicities since they do not need to be as potent as conventional chemotherapeutic agents.\(^\text{180}\)
Gangjee et al.\textsuperscript{181-184} have reported the discovery of single agents with dual antiangiogenic and cytotoxic activities. The antiangiogenic effects of these compounds were due to inhibition of RTKs, and the cytotoxic effects were due to inhibition of dihydrofolate reductase\textsuperscript{181} or thymidylate synthase\textsuperscript{183} or an unknown target.\textsuperscript{184} For cytotoxic effects, tubulin was chosen as the main target as several clinical and preclinical combinations of tubulin inhibitors and VEGFR2 inhibitors have been successful.\textsuperscript{185-187} For a variety of different cancers including colorectal, breast, lung among others, several clinical trials are ongoing that involve FDA-approved antiangiogenic agents as well as those in development, and cytotoxic chemotherapeutic agents, and include microtubule targeting agents (MTAs) CA4P, paclitaxel, docetaxel, vincristine, vinblastine with antiangiogenic agents bevacizumab, axitinib, sorafenib, sunitinib, cediranib, pazopanib, vandetanib and vatalanib.\textsuperscript{188} Additionally, half of all human tumors have mutations in the p53 gene, and p53 status effects vulnerability of tumor cells to chemotherapy via cell cycle arrest. The most effective drugs in cell lines with p53 gene mutations are MTAs.\textsuperscript{189-190}

Several highly potent MTAs have failed in the early stage clinical trials due to toxicities. Discodermolides and cryptophycin 52 (LY355703) are recent examples of such agents.\textsuperscript{191-192} All clinically used MTAs share neurological toxicity as the most prominent dose-limiting side effect.\textsuperscript{5} Other common toxicities of microtubule-binding agents include myelosuppression and neutropenia. Neutropenia was observed in several combination chemotherapy clinical trials with other drugs and was mostly manageable.\textsuperscript{193-195} The myeloid toxicity, which is usually reversible, is seen with vinca alkaloids and taxanes.\textsuperscript{196-}
Thus, new agents are needed which are safe and effective, and have both antiangiogenic and cytotoxic activities in a single agent.

Figure 22. Hybrid design from lead compounds

7-Benzyl-pyrrolo[2,3-\(d\)]pyrimidines with general structure 122 (Figure 22) have been reported by Gangjee et al.\(^\text{198}\) as antiangiogenic, antimetastatic and antitumor agents. To engineer RTK inhibitory activity in 121·HCl, without loss of MTA activity, hybrid 129·HCl was designed that incorporates the 7-benzyl group of 122 onto the pyrrolo[3,2-\(d\)]pyrimidine scaffold of 121·HCl. This analog 129 was reported recently by Gangjee et al.\(^\text{180}\)

Compound 129·HCl maintained cytotoxic antitubulin activity of 121·HCl, and improved activity against VEGFR2 by 9-fold for antiangiogenic effects over 121·HCl (Figure 22). Compound 129·HCl has an inhibitory potency against VEGFR comparable with the clinically used sunitinib and clinically evaluated semaxinib. The cytotoxic effect, mediated by the microtubule targeting, was independent of overexpression of Pgp and \(\beta\)III-tubulin
(two clinically relevant tumor resistance mechanisms). Thus, \textbf{129}\textsuperscript{·}HCl has combination chemotherapeutic potential in a single agent.\textsuperscript{180, 199}

The general RTK pharmacophore model consists of an Adenine region, a Sugar binding pocket and a Phosphate binding region which binds the adenine ring, the sugar moiety and the triphosphate moiety of ATP respectively. Additionally, there are two hydrophobic regions I and II, neither of which are used by ATP for binding. The 7-benzyl group in \textbf{129}\textsuperscript{·}HCl could access the Sugar binding pocket as shown in binding mode 1 (Figure 23). Alternatively, the pyrrolo[3,2-\(d\)]pyrimidine compounds could adopt mode 2 in which the compounds are rotated around the 2-\(CH_3\)-C2 bond. In this mode, the 7-benzyl group and the 4-anilino groups could occupy Hydrophobic region I and Sugar binding pockets respectively. These compounds could also adopt binding mode 3 in which the molecule is rotated by 60\(^\circ\) (from mode 2). In this mode, the 7-benzyl group could orient towards the Hydrophobic region I and the 4-anilino groups could occupy Hydrophobic region II.
Figure 23. Predicted binding modes of $129 \cdot \text{HCl}$ in VEGFR2 [pdb: IYWN]$^{200}$
Design of single agents with combination chemotherapy and multiple RTK inhibitory potential

The complexity of the angiogenic pathways implies that disrupting only a single mechanism of angiogenesis may not result in long term clinical success. Multiple RTKs are co-activated in tumors and redundant inputs drive and maintain downstream signaling, thereby limiting the efficacy of therapies targeting single RTKs. Resistance to anti-VEGF treatment is associated with increased PDGFR expression within the tumor, increased recruitment of pericytes to tumor vasculature, and increases in other proangiogenic factors. Preclinical models suggest that PDGF-mediated recruitment of pericytes may contribute to resistance to VEGF blockade. EGFR inhibition can lead to VEGFR2 up-regulation which subsequently promotes tumor growth signaling independent of EGFR and thus contributes to the resistance of EGFR inhibitors. The effect of EGFR inhibition can also be partially overcome by activation of PDGFR signaling and the subsequent transactivation of HER-3 (EGFR family) signaling to promote alternate tumor growth signaling. Hence, targeting multiple RTKs maximizes the proportion of angiogenic signaling that is effectively targeted. Moreover, high intratumoral heterogeneity has been reported with different subpopulations producing distinct growth factors. Targeting a single RTK could be ineffective due to subpopulations of cells that are either not affected by the drug mechanism and possess or acquire a greater drug resistance. Hence, it was of interest to explore the effect of structural variation and its effect on activity against the RTKs VEGFR2, PDGFRβ and EGFR in addition to having
cytotoxic antitubulin effects. The aim of this work was to identify single agents with antitubulin and multiple RTK inhibitory potential.

Thus, it was of interest to elucidate the effect of conformational flexibility on antiangiogenic/MTA activities to achieve multikinase inhibition. Therefore, **Series I** (Figure 24) was designed as dual antiangiogenic and microtubule targeting agents with multikinase inhibition potential.

![Figure 24. Series I](image)

Compounds 130 and 131 were designed as open chain analogs of 129·HCl. Compounds 130 and 131 would be conformationally flexible compared to lead 129·HCl, and so will have more freedom to bind in different binding modes to different targets which could afford multikinase inhibition. Compound 132 would be a more rigid structure than 130 or 131, and was designed to determine the effect of conformational restriction on the inhibitory activity of 130 and 131.
Figure 25. Distances between pyrimidine and phenyl (between carbons a and b, Figure 24) for 129·HCl and 130-132.

Figure 25 shows analogs 130-132 in their minimized forms using MOE2016.08. Distances between the pyrimidine and the side-chain phenyl (between carbons a and b, Figure 25) for 129·HCl and 130-132 were calculated. Compounds 131 and 132 could extend further in the binding pocket of multiple RTKs, and access the hydrophobic residues of RTKs better than 129·HCl as shown in Figures 27a-c.
**Figure 26.** Docked pose of 130 (green) overlaid with 129 (brown) in the colchicine site (PDB ID 4O2B).

**Docking studies:** Compound 130 was docked in the colchicine site of tubulin (PDB: 4O2B, 2.3 Å)\(^{209}\) using MOE2016.08\(^{183}\) (validated by re-docking the crystal structure ligands) to predict the potential binding of these analogs (Figure 26). The docked conformation of 129 in the colchicine site of tubulin is based on its similarity to the bound conformation of colchicine\(^{210}\) (not shown in the model). It was observed that 130, open chain conformationally flexible analog of 129, binds in a similar manner as 129. The 4'-OMe phenyl group of 130 is oriented towards the triOMe containing A-ring of colchicine and interacts with Cys241 and HOH615. The N-Me of 130 is involved in hydrophobic interactions with Leu255 and Ala250. 2-Me is involved in hydrophobic interactions with Ala354 and Ala316. The 6-benzyl moiety makes hydrophobic interactions with Val181 and Phe404. Compound 130 has a docked score of -6.34 kcal/mol, similar to that of the
lead $129 = -6.57$ kcal/mol. This suggested that $130$ should retain activity against tubulin as $129$ did.

**Figure 27a.** Docked pose of $130$ (green) overlaid with $129$ (brown) in the ATP site of VEGFR-2 (PDB ID 1YWN). $^{211}$

Compound $130$ was also docked in the ATP site of VEGFR2 (PDB: 1YWN, 1.75 Å). $^{211}$ Using MOE2016.08. $^{183}$ Figure 27a shows the docked pose of $130$ and $129$ in the ATP site of VEGFR2. In the best pose of $130$ (docked score of -7.02 kcal/mol), the phenyl 4′-OMe is observed in the same location as the benzyl moiety of $129$, and interacts with Leu1047 and Ala1048. The benzyl moiety of $130$ is oriented towards Val897 and Ile1042. It was observed that $130$, because of its extra flexibility, could bind in multiple poses and, could
provide multikinase inhibition. Compound 129 because of the constraint imposed by the second ring, did not afford multiple poses.

Due to the issues with the MOE server in the chemistry department of Duquesne University during the final parts of the dissertation writing, rest of the modeling in EGFR and PDGFRβ modeling was done using LeadIT2.1.3 and images were taken directly from there.

![Docked pose of 130 in the ATP site of EGFR (PDB ID 4WKQ).](image)

**Figure 27b.** Docked pose of 130 in the ATP site of EGFR (PDB ID 4WKQ).²¹²
Compound 130 was docked in the ATP site of EGFR (PDB: 4WKQ, 1.85 Å)\textsuperscript{212} and PDGFRβ (homology model) using LeadIT2.1.3 (Figures 27b and 27c). In the EGFR crystal structure, the phenyl 4′-OMe is observed interacting with Ala743 and Ile 744. The benzyl moiety of 130 is oriented towards the long side chain of Lys745. 2-Me is oriented towards Ser70. The best docked pose of 130 had a score of $-16.12 \text{ kJ/mol}$. In the PDGFRβ homology model (docked pose of $-15.56 \text{ kJ/mol}$), the aniline is observed interacting with Val631 and Lys630. The anilino moiety of 130 is flipped in this case compared to that in EGFR or VEGFR. The benzyl moiety of 129 is oriented towards Val615 and 2-Me towards Ala632.
Figure 28. Compound 122 was synthesized as an intermediate.

Table 1. Effects of 122 on tubulin, and inhibition of colchicine binding compared to 121·HCl.

<table>
<thead>
<tr>
<th>R</th>
<th>Inhibition of tubulin assembly IC$_{50}$ (µM) ± SD</th>
<th>Inhibition of colchicine binding (% Inhibition ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 µM inhibitor</td>
</tr>
<tr>
<td>CA4</td>
<td>0.96 ± 0.07</td>
<td>89 ± 0.6</td>
</tr>
<tr>
<td>121·HCl</td>
<td>10 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>122</td>
<td>µ</td>
<td>63 ± 1</td>
</tr>
</tbody>
</table>

Compound 122 was an important intermediate in the synthesis of 130. Because 122 is an open chain, conformationally flexible analog of 121·HCl, docking studies were done to predict its potential binding modes.
Figure 29a. Docked pose of 122 (green) overlaid with 121 (brown) in the colchicine site (PDB ID 4O2B).

**Docking studies:** Compound 122 and 121 were then docked in the colchicine site of tubulin (PDB: 4O2B\(^{209}\), 2.3 Å) using MOE2016.08\(^{183}\) (validated by re-docking the crystal structure ligands) to predict potential binding of these analogs for docking studies (Figure 29a). The docked conformation of 121 in the colchicine site of tubulin is based on its similarity to the bound conformation of colchicine, not shown in Fig. 29a. It was observed that 122 binds in similar fashion as 121. The 4′-OMe phenyl group of 122 is oriented towards the triOMe containing A-ring of DAMA-colchicine and interacts with Cys241 and HOH728. The N-Me of 122 is involved in hydrophobic interactions with Leu255 and...
Ala250. The 2-Me is involved in hydrophobic interactions with Ala354 and Ala316. Compound 122 has a docked score of -6.62 kcal/mol, which is similar to the docked score of 121: -6.95 kcal/mol.

Figure 29b. Docked pose of 122 in the ATP site of EGFR (PDB ID 4WKQ).  

Compound 122 was also docked in the ATP site of EGFR (PDB: 4WKQ, 1.85 Å) and PDGFRβ (homology model) using LeadIT2.1.3 (Figures 29b and 29c). In the EGFR crystal structure, the anilino moiety is observed interacting with Leu792 and Pro794. The 4N-Me of 122 is oriented towards the long side chain of Gly796 and Phe795. The best docked pose of 122 had a score of −5.25 kcal/mol. In the PDGFRβ homology model, the aniline is observed interacting with Tyr683 and Lys630. The 2-Me is oriented towards Gly616 and Val615. The best docked pose of 130 had a score of −5.94 kcal/mol.
Because 122 is an open chain, conformationally flexible analog of 121·HCl (Figure 28), its effects on the polymerization of purified bovine brain tubulin was evaluated (Table 1), and compared with CA4 and 121·HCl. CA4 is the active metabolite of the water soluble prodrug combretastatin A-4 phosphate (Zybrestat, Fosbretabulin), which has orphan drug status for ovarian cancer. Interestingly, 122 was found to be a potent inhibitor of bovine tubulin assembly with about 5-fold greater inhibitory activity than the parent 121·HCl, and only 2-fold less inhibitory activity than CA4. The best docked pose of 122 and 121 had scores of $-15.99 \text{ kJ/mol}$ and $-15.61 \text{ kJ/mol}$.
Table 2. RTK and CAM inhibitory activities (IC<sub>50</sub> ± SD)

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Kinase inhibition IC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
<th>CAM angiogenesis inhibition IC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGFR</td>
<td>VEGFR-2</td>
</tr>
<tr>
<td>121·HCl</td>
<td>29.5 ± 3.1</td>
<td>182.3 ± 20.6</td>
</tr>
<tr>
<td>122</td>
<td>415.4 ± 50.1</td>
<td>33.2 ± 4.1</td>
</tr>
<tr>
<td>PD153035</td>
<td>0.21 ± 0.002</td>
<td>-</td>
</tr>
<tr>
<td>SU5416</td>
<td>-</td>
<td>12.9</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
</tr>
</tbody>
</table>

Although, 122 was not initially designed as an antiangiogenic agent, its potent activity against bovine tubulin assembly prompted us to evaluate its antiangiogenic effects in keeping with our aim of providing multitargeted agents. In VEGFR2 expressing cell line, it was determined that 122 was 5-fold more inhibitory compared to 121·HCl with 2-fold less potency than the standard sunitinib (Table 2). These results are remarkable as VEGFR2 is the principal mediator of angiogenesis. In PDGFRβ inhibitory assay, 122 was 1.5-fold more active than 121·HCl. These results indicated that the open chain monocyclic, flexible pyrimidines not only preserve but increase dual potency against both targets tubulin and multiRTK.
Compound 122 (with 6-Cl) was an intermediate in the process of synthesizing 130 (6-benzyl). The compound turned out to be an active compound in tubulin depolymerization assays as well as kinase inhibition assays. To further increase the activity by utilizing hydrophobic interactions with the hydrophobic residues in the active site, homologation of N5 of 122 was done, and 123-127 were designed.

The discovery of 122 as a dual tubulin and RTK inhibitor prompted a SAR study, and Series II (Figure 30) was designed to address the effect of homologation at N5 of 122 (compounds 123, 125-127) and chain branching (124,128) on potency at both tubulin and RTK sites.

1. With this series, optimum chain length of the N5-substituent could be determined. This would effectively mimic the pyrrole ring of 121·HCl.

2. The biological activities of Series II will allow a determination of the effect of increased hydrophobicity on tubulin, EGFR, VEGFR2 and PDGFRβ inhibition, and provide a SAR of N5-substituents for inhibition of tubulin and multiple RTKs.
3. Chain branching as in iBu makes the alkyl chain more compact, and cause less disruption of hydrogen bonding network of water, and can cause major changes in pharmacological activity.\textsuperscript{213}

4. Increased lipophilicity of the molecule due to the increased chain length may also permit better penetration into the cell membranes, and might be leading to better activities in cell assays.

5. With compounds 123-127, we are utilizing these hydrophobic interactions:
   Hydrophobic residues in tubulin site - Ala316, Val315, Val181 and Ile378.
Figure 31. Docked pose of 125 (green) overlaid with 121·HCl (brown) in the colchicine site (PDB ID 4O2B).

Compound 123-128 were docked in the colchicine site of tubulin (PDB: 4O2B\textsuperscript{209}, 2.3 Å) using MOE2016.08\textsuperscript{183} (validated by re-docking the crystal structure ligands) to predict potential binding of these analogs for docking studies. The docked conformation of 121·HCl (Figure 31) in the colchicine site of tubulin is similar to colchicine. It was observed that 125 binds in a similar fashion as 121·HCl, and mimics the pyrrole ring of 121·HCl better than 122.

The N-ethyl chain extends to the hydrophobic pocket consisting of Ala316, Val315, Val181 and Ile378. Compound 125 has a docked score of -7.29 kcal/mol, which is better
than that of the lead 121·HCl: -6.95 kJ/mol, and 122: -6.62 kcal/mol because of the extra hydrophobic interactions.

Figure 32a Docked pose of 125 (green) overlaid with 121·HCl (brown) in the ATP site of VEGFR-2 (PDB ID 1YWN).211

 Compound 123-128 were also docked in the ATP site of VEGFR2 (PDB: 1YWN, 1.75 Å).211 Figure 32a shows the docked pose of 125 in the ATP site of VEGFR2. The pyrimidine scaffold of 125 forms hydrophobic interactions with Lys866, Val914 and Cys1040. The N4-methyl forms hydrophobic interactions with Leu838 and Val846. The
4′-OMe of 125 is oriented towards Leu1047 and Ala1048. The best docked pose of 125 had a score of −6.85 kJ/mol.

Figure 32b. Docked pose of 127 (green) overlaid with 121·HCl (brown) in the ATP site of VEGFR-2. PDB: 3BE2.²₁²

Figure 32b shows the docked pose of 127 in the ATP site of VEGFR2 (PDB: 3BE2²₁², 1.75 Å) using LeadIT2.1.8. The pyrimidine scaffold of 127 is oriented parallel to the hinge region amino acids, and occupies the adenine binding region of the ATP site of VEGFR2. The N1 nitrogen of 127 forms a hydrogen bond interaction with the hydroxy group of Thr916. 2-methyl interacts with Ile1044. The 4′-OMe of 127 interacts with Phe845. The 4-anilino ring interacts with Phe1047. The 4N-methyl is oriented towards the hydrophobic
site formed by Val848. The propyl chain at N5 of 127 is involved in hydrophobic interactions with Leu840 and Leu1035. The best docked pose of 127 and 121 had scores of $-17.27$ kJ/mol and $-17.51$ kJ/mol.

Figure 32c. Docked pose of 127 (yellow) overlaid with 121 (green) in the ATP site of EGFR (PDB ID 1M17). $^{214}$

Figure 32c shows the docked pose of 127 in the ATP site of EGFR (PDB: 1M17 – 2.6Å). $^{214}$ The pyrimidine scaffold of 127 forms hydrophobic interactions with Leu820, Leu694. The N4-methyl forms hydrophobic interactions with Val702. The 4'-OMe of 127 is oriented towards Lys721 and Phe832. The 5N-butyl chain is oriented towards Ala719, Thr830 and
Thr766. The best docked pose of \textbf{127} and \textbf{121} had scores of -17.13 kJ/mol and -16.74 kJ/mol.

\textbf{Figure 32d.} Docked pose of \textbf{127} (yellow) overlaid with \textbf{121} (green) in the homology model of PDGFR\textgreek{b}.

Figure 32d shows the docked pose of \textbf{127} in the homology model of PDGFR\textgreek{b}. The N4-methyl forms hydrophobic interactions with Ala848 and Phe845. The 4'-OMe of \textbf{127} is oriented towards Leu606. The 5N-butyl chain is oriented towards Val665 and Phe845. The best docked pose of \textbf{127} and \textbf{121} had scores of -16.71 kJ/mol and -16.99 kJ/mol.
Thus, the molecular modeling studies provided support for the synthesis and biological evaluation of 123-128.

**Results found:**

So, in sum, with compounds 123-127, we are utilizing these hydrophobic interactions: Hydrophobic residues in tubulin site - Ala316, Val315, Val181 and Ile378. Hydrophobic residues in VEGFR site Leu840 and Leu1035. Hydrophobic residues in EGFR site Ala719. Hydrophobic residues in PDGFR homology model Val665 and Phe845.

Replacement of 6-Cl of 122 with 6-Me was not tolerated and so 6-Cl was not replaced for further target compounds. Compared to the pyrrolo[2,3-\(d\)]pyrimidine compound 121, the lone pair of 5-NH\(_2\) in 122 is not part of the ring system and so available for hydrogen bonding with residues in the active site of various targets as a hydrogen bond donor as well as acceptor. Compound 121 doesn’t have a hydrogen bond acceptor due to the nitrogen lone pair being part of the pi system. Compound 122 NH\(_2\) lone pair might be involved in conjugation with the pyridine ring but still can form hydrogen bond with the residues at the active site.
Also, 123 with a 6-Cl might be more active than 121 because of the electron withdrawing nature of the 6-chloro group in 122. Any electron donating group at 6-position like OH, NH₂ or alkyl groups were not tolerated. Replacement of Cl with similar bulk Me was also not tolerated. But replacement with other electron withdrawing groups at 6-position like trifluoromethyl was tolerated. So, we believe, it is because of the electron withdrawing nature of the Cl, or 5-NH₂ being involved in hydrogen bonding as a hydrogen bond acceptor (although it is a hydrogen bond donor too), which is leading to the improved activity compared to 121.

In molecular modelling studies, compound 122-128 bound better in the crystal structure of target protein than the lead compound 121, and had better scores (about 1-2 units higher).

**Design rationale for Series III**

![Chemical structure](image)

**Figure 33. Series III**

**Table 3.** tPSA and cLogP of 122 and 133-136.

<table>
<thead>
<tr>
<th>Cmpd.</th>
<th>tPSA (Å²)</th>
<th>CLLogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>63.21</td>
<td>3.5</td>
</tr>
<tr>
<td>133</td>
<td>66.29</td>
<td>2.8</td>
</tr>
<tr>
<td>134</td>
<td>74.57</td>
<td>2.7</td>
</tr>
<tr>
<td>135</td>
<td>66.29</td>
<td>4.1</td>
</tr>
<tr>
<td>136</td>
<td>89</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Series III was designed to determine the effects of electron withdrawing N-acetyl, N-pivylyl, and nitro group in the 5 position of pyrimidine ring on the biological activity (Figure 33). The polar amide bond in the acetyl and pivalic groups is capable of forming a hydrogen bond because of the presence of both donor as well as acceptor moieties. Figure 34 shows pivalic carbonyl oxygen of 135 forming a water-mediated (HOH623) hydrogen bond with the carbonyl of Thr353. Table 3 shows the calculated LogP as well as tPSA (Topological Polar Surface Area) values of 133-136 (calculated using ChemBioDraw Ultra 15.1.0.144). Compound 133 with N-acetyl would be less lipophilic (Table 3), and 135 with N-pivalic group would be more lipophilic than 122. The nitro moiety at the 5-position (136) has highest tPSA.

Figure 34. Docked pose of 135 (green) in the colchicine site (PDB ID 4O2B). Surface: black hydrophilic and red lipophilic.
Table 4. Effects of 121·HCl on tubulin and VEGFR2 in cellular assays

<table>
<thead>
<tr>
<th></th>
<th>Inhibition of bovine tubulin assembly IC₅₀ (µM) ± SD</th>
<th>MDA-MB-435 IC₅₀ ± SD (nM)</th>
<th>VEGFR2 kinase inhibition IC₅₀ ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>121·HCl</td>
<td>10 ± 0.6</td>
<td>96.6 ± 5.3</td>
<td>183 ± 3.4</td>
</tr>
<tr>
<td>CA4</td>
<td>0.96 ± 0.07</td>
<td>3.47 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>-</td>
<td>-</td>
<td>18.9 ± 2.7</td>
</tr>
</tbody>
</table>

Table 5. Compound 121·HCl overcomes overexpression of Pgp and βIII-tubulin.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC₅₀ ± SD in HeLa cells (nM)</th>
<th>IC₅₀ ± SD in WTβIII (nM)</th>
<th>Rr value</th>
<th>IC₅₀ ± SD in SK-OV-3 cells (nM)</th>
<th>IC₅₀ ± SD in SK-OV-3 MDR1-M6/6 cells (nM)</th>
<th>Rr value</th>
</tr>
</thead>
<tbody>
<tr>
<td>121·HCl</td>
<td>179 ± 8.7</td>
<td>158 ± 13</td>
<td>0.8</td>
<td>219 ± 40</td>
<td>448 ± 110</td>
<td>2.1</td>
</tr>
<tr>
<td>CA4</td>
<td>5.6 ± 0.3</td>
<td>5.7 ± 0.4</td>
<td>1.0</td>
<td>6.9 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.9 ± 0.3</td>
<td>32.9 ± 5.8</td>
<td>17.3</td>
<td>6.3 ± 0.4</td>
<td>1,187.2 ± 265.2</td>
<td>188</td>
</tr>
</tbody>
</table>

Efforts to elucidate the plausible binding modes of multiple RTK-inhibitors led Gangjee *et al.* 215 to the discovery of a highly potent water-soluble antitumor pyrrolo[2,3-d]pyrimidine 121·HCl (Figure 22). 216-217 It was discovered to be a colchicine site binding, microtubule depolymerizing agent that inhibited the growth of cancer cells with IC₅₀ in the nanomolar range (Table 4). The effects of 121·HCl were evaluated in two pairs of multidrug resistant cell lines (Table 5). Compound 121·HCl was evaluated in the parental HeLa cells and in an engineered cell line overexpressing βIII tubulin (WTβIII). In addition, 121·HCl was
evaluated for their ability to inhibit the growth of SK-OV-3 cells overexpressing Pgp in comparison to the parental line.\textsuperscript{218-222}

![Figure 35. Series IV](image)

It was of interest to identify the structural features that are crucial for tubulin and RTK inhibition. Hence, 137-139, conformationally flexible, open chain analogs of 121-HCl were designed to elucidate the minimal structural requirements to retain tubulin and RTK inhibition (Series IV, Figure 35). The methyl and ethyl groups in the open chain analogs 138 and 139 mimic the pyrrolo ring of pyrrolo[3,2-$d$]pyrimidine 121-HCl.

Both pyrrolo[2,3-$d$]pyrimidine\textsuperscript{223} and pyrrolo[3,2-$d$]pyrimidine\textsuperscript{224} derivatives have been reported to possess potent inhibitory activity against tubulin. Hence, 140 with amino at both 5- as well as 6-position was designed.
Figure 36. Docked pose of 140 (green) overlaid with 129·HCl (brown) in the colchicine site (PDB ID 402B) showing the distance between 6-amino protons and Asn258 side chain.

The amino protons at 6-position interact with Asn258 side chain amide oxygen with a hydrogen bond (Figure 36, distance calculated using MOE2016.08 = 2.74 Å).

Figure 37. Series V
To determine whether the excellent tubulin/VEGFR-2 dual activity of compound 122 is due to the electron withdrawing nature of the 6-chloro group in 122, Series V (Figure 37) was designed to evaluate the effect of electron-withdrawing substituents at 6-position by substituting the 6-chloro group in 122 with electron withdrawing groups, -CN, -I, and –CF₃, 141-143 respectively.

**Table 6.** tPSA and cLogP of 122 and 141-143

<table>
<thead>
<tr>
<th>Cmpd.</th>
<th>cLogP</th>
<th>tPSA (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>3.53</td>
<td>63.21</td>
</tr>
<tr>
<td>141</td>
<td>2.51</td>
<td>87</td>
</tr>
<tr>
<td>142</td>
<td>3.64</td>
<td>63.21</td>
</tr>
<tr>
<td>143</td>
<td>3.73</td>
<td>63.21</td>
</tr>
</tbody>
</table>

In series IV, electron donating groups like alkyl and amino groups were designed but the compounds turned out to be less active/ inactive. 6-hydroxy has already been synthesized in our group and was found to be inactive. Because of this reason, in series V, electron withdrawing groups (lipophilic and hydrophilic) were designed.

The biological activities of compounds 141-143 will allow a determination of the effect of electronics and size on EGFR, VEGFR2 and PDGFRβ as well as tubulin inhibition. Table 6 shows the calculated LogP as well as tPSA values of 141-143 (calculated using ChemBioDraw Ultra 15.1.0.144). Compound 141 is less lipophilic compared to 122 with a higher polar surface area (Table 6). The nitrile moiety is more electron withdrawing compared to the chloro moiety in 122.²¹³ Compound 142 would be more lipophilic and
bulkier than 122. The iodo in 142 also has the potential to form a stronger halogen bond compared to a chloro.\textsuperscript{225}

Compound 143 with a lipophilic trifluoromethyl moiety would be a stronger electron withdrawing group than the chloro group due to the presence of three fluroine atoms, thereby, altering the electronics of the pyrimidine ring system making it more electron poor.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure38.png}
\caption{Series VI}
\end{figure}

To evaluate the importance of the 2-methyl of 122, Series VI (Figure 38) was designed by replacing the 2-methyl with different groups. To determine, if the 2-methyl is specifically required for hydrophobic interactions, compounds 144 (desmethyl) and 145-147 (2-ethyl) were designed. These compounds would evaluate the steric effect at the 2-position on biological activity.

Gangjee \textit{et al.}\textsuperscript{226} have reported that a 2-amino group on fused pyrimidine ring systems improves hinge region binding and provides multiple-RTK inhibition. Compounds 148-150 (Figure 38) were designed by replacing the 2-methyl group in 122 with a 2-amino
group which could potentially increase binding to the hinge region of EGFR or PDGFRβ via hydrogen bonds, without loss of tubulin and VEGFR2 inhibitory activities.

**Figure 39. Series VII**

Series VII (Figure 39) addresses the effect of bioisosterism, conformational restriction and flexibility on biological activity by introducing various substituted anilines in the 4-position of the pyrimidine ring of 122 in place of the 4′-OMe aniline.

**Figure 40. Design rationale and biological activity for 301.**
When the 4’-methoxy group of 301 was replaced with a 4’-thiomethyl group (Figure 40), it led to a 2-fold increase in activity against tubulin, and a 1.5-fold increase in inhibition of cell proliferation. Therefore, 151 with a 4’-thiomethyl group (Series VII) was designed.

![Chemical structure](image1)

**Figure 41.** Dihedral angle $\alpha$: C5-C4-4N-C1’ (bond rotation about bond a)

![Chemical structure](image2)

**Figure 42.** Dihedral angle $\beta$: C5-C4-C1’-C2’ (bond rotation about bond b)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dihedral angle α</th>
<th>Dihedral angle β</th>
<th>Compound</th>
<th>Dihedral angle α</th>
<th>Dihedral angle β</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>45.7</td>
<td>78.3</td>
<td>151</td>
<td>48.1</td>
<td>65.0</td>
</tr>
<tr>
<td>152</td>
<td>50.7</td>
<td>65.2</td>
<td>153</td>
<td>44.9</td>
<td>78.3</td>
</tr>
<tr>
<td>154</td>
<td>176.9</td>
<td>47.7</td>
<td>155</td>
<td>172.2</td>
<td>45.8</td>
</tr>
</tbody>
</table>

Bond rotations about 2 rotatable single bonds, b and c in the lead molecule 122 (Figure 39) were restricted in compounds 152 and 153 (Series VII), respectively, thus, affording a much more rigid structure than 122 but still maintaining the phenyl and alkyl substitutions on the N4 as in 122. This substitution could also restrict the rotation around the N-phenyl bond, thus providing conformational rigidity and perhaps improved potency.

Dihedral angles α (Figure 41) and β (Figure 42) were calculated for 122 and compounds 151-155 using MOE2016.08 in their energy minimized conformations. The results clearly tell that compounds 154 and 155 without 4N-Me have a higher dihedral angle α suggesting that phenyl is not on top of the 5-amino but on the other side.

Conformational restriction of the phenyl and 4′ methoxy moieties in 152 and 153 respectively could lock the compound in a conformation that is conducive to selective RTK (VEGFR2 or other) inhibition without loss of tubulin inhibitory activity. Conformational
restriction of the flexible 4-anilino moiety of 122 would minimize the entropic loss associated with the 4-anilino moiety adopting the preferred conformation for binding to a specific RTK. This could enhance potency for RTK and/or tubulin.\(^{227}\)

Compounds 154 and 155 (Series VII) were designed to evaluate the importance of the 4-NCH\(_3\), and to increase the free rotation about the 4-position C-N bond (bond a) as well as the 1’-position C-N bond (bond b) in 122 and 151, respectively.

Rationale explained further: As phenyl ring of 122 can exist in multiple conformations, this can result in unexpected entropic costs while binding to the colchicine bind site of tubulin. High entropic cost would offset any possible enthalpic gains that may have been achieved while binding to the active site. Hence, compounds 152 and 153 were designed as conformationally restricted analogs of 122 restricting the rotation about the bonds b and c respectively (Fig. 39, pg 87). A series of restricted analogs have already been designed and synthesized for other scaffolds like thieno[2,3-\(d\)]pyrimidines and pyrrolo[2,3-\(d\)]pyrimidines, and it is found that the replacement of 4’-methoxyaniline moiety with bicyclic 6-methoxy-tetrahydroquinoline (152) and 4-N-methyl- 2’-3’-dihydrobenzofuran moiety (153) are the most active as they maintain the phenyl and alkyl substitution at N4 of 122. Also, the 4-methoxy group of 152 and 153 maintained the hydrogen bonding interaction with Cys241 in tubulin binding site.

Compound 151 was designed as a bioisostere of 122 by replacement of 4’-methoxy group with a 4’-thiomethyl group to determine the bulk tolerance (O vs S) as well as the
importance of hydrogen bonding. Sulfur being a bulkier hydrophobic group could increase the activity by increasing the hydrophobic interactions at the colchicine site.

The S-Me compound 151 was also designed because it showed improved activity in other scaffolds. For instance, when the 4’-methoxy group of 300 (Fig. 40) was replaced with a 4’-thiomethyl group (301), it led to a 2-fold increase in activity against tubulin, and a 1.5-fold increase in inhibition of cell proliferation. Therefore, 151 with a 4’-thiomethyl group was designed.

The N-Me of 122 is involved in hydrophobic interactions with Leu255 and Ala250 at the colchicine binding site of tubulin. To gain the kinase inhibitory activity by improving hydrogen bonding interactions with the hinge region, N-methyl at the 4-position of 122 was replaced with the N-H (compounds 154 and 155) that would provide additional H-donor binding in RTK active sites. 4N of 154 and 155 would still maintain its hydrogen bond acceptor capabilities. This in turn would make it possible to inhibit multiple RTKs. Moreover, in RTK inhibitors, such as gefitinib, erlotinib, lapatinib, N-H is involved in hydrogen bonding interaction with the kinase region in multiple RTKs. Same strategy has been used while designing 154 and 155.

Omitted compounds-

The SAR for tubulin inhibition has already been established for other scaffolds in Dr. Gangjee’s group. Meta and ortho compounds have already been designed and synthesized before for other scaffolds. It was concluded with the biological results that only para-methoxy group is active for tubulin depolymerization as it can form a hydrogen bond.
interaction with Cys241 in the colchicine binding site of tubulin while meta and ortho analogs cannot. A series of conformationally restricted analogs have already been designed and synthesized for other scaffolds like thieno[2,3-d]pyrimidines and pyrrolo[3,2-d]pyrimidines, and it is found that the replacement of 4'-methoxyaniline moiety with bicyclic 6-methoxy-tetrahydroquinoline and 4-N-methyl- 2'-3'-dihydrobenzofuran moiety lead to the most active analogs as they maintain the phenyl and alkyl substitution at N4 of 122. Also, the 4-OR of 152 and 153 maintained the hydrogen bonding interaction with Cys241 in tubulin binding site.

Homologation of 4N has been reported for other scaffolds but it was found to be detrimental for the inhibitory activity.

**Design rationale for 4,6-disubstituted compounds**

![Design rationale for 156.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tubulin IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>VEGFR-2 IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>129.HCl</td>
<td>21 ± 1 μM</td>
<td>21.3 ± 3.2 nM</td>
</tr>
<tr>
<td>156</td>
<td>&gt; 20 μM</td>
<td>7.9 ± 1.8 nM</td>
</tr>
</tbody>
</table>

**Figure 43.** Design rationale for 156.
The potent inhibitory activity of 129·HCl, a N4-,7-disubstituted analog, in cell lines overexpressing VEGFR2 led to the design of 156 (Figure 43) with 4′-methoxy-N-methylaniline in the 6-position to determine its effects on the cell lines overexpressing multikinases. The 6-benzyl moiety in 129·HCl occupies hydrophobic region I or sugar binding pocket in different modes (described above, Figure 23). Similarly, the 4′-methoxy-N-methylaniline moiety at the N6-position of 156 could also access different pockets in the ATP binding site to afford multikinase inhibition.

**Figure 44.** Docked pose of 156 (green) overlaid with 129·HCl (brown) in the ATP site of VEGFR-2 (PDB ID 1YWN).²¹¹

**Docking studies:** Figure 44 shows the docked pose of 156 and 129·HCl in the ATP site of VEGFR2 (PDB: 1YWN, 1.75 Å).²¹¹ In the best docked pose of 156 (docked score of -7.94
kcal/mol), the 4′-methoxy-N-methylaniline at the 6-position occupies the site where the benzyl moiety of 129·HCl binds, and interacts with Leu1047 and Ala1048. The 4′-OMe of the N6 substitution of 156 is oriented towards Arg840 and could involve a hydrogen bonding interaction.

Compound 156 was found to have single digit nanomolar inhibition in cell lines overexpressing VEGFR2 and was greater than a 4-fold increase in activity compared to lead 129·HCl albeit with loss of inhibition of tubulin activity.

Figure 45. Series VIII
Because of the loss of tubulin activity in 156, Series VIII (Figure 45) with 157-160 was designed to regain the lost tubulin inhibitory activity while retaining or further improving the potent VEGFR2 inhibitory activity.

Compounds 157 and 158 without N-methyl can have hydrogen bonding interactions with the tubulin protein in the colchicine binding site due to two additional hydrogen bond donors, one at the N4 and the other at the N6. This might restore the tubulin inhibitory activity.

**Figure 46.** General pharmacophore model of pyrimidines with potential binding mode of 157.

In addition, binding modes of 157 and 158 with N4-H and N6-H are proposed (Figure 46) to enable inhibition of an increased spectrum of RTKs compared to 156. In an attempt to
design similar multikinase inhibitors, a general RTK pharmacophore model was used as opposed to a specific crystal structure of a specific RTK to approach multikinase inhibition. It was envisioned that removing the N-methyl at the 4- and 6-positions would provide additional hydrogen bond donors which could provide additional H-donor binding in RTK active sites in multiple binding modes relative to the corresponding N-methyl compounds. This in turn would make it possible to inhibit multiple RTKs. Compounds 159 and 160 were designed to regain the lost tubulin inhibitory activity by the replacement of 4´-OMe at 4- and 6-positions of 156 with 4´-SMe, and 4,6-bis-4´methoxyanilino moiety with 4,6-bis-6´-methoxy-3,4-dihydroquinolino moiety respectively.

**Design rationale for Series IX**

![Design rationale for Series IX](image_url)

**Figure 47. Series IX**
Gangjee et al.\textsuperscript{228} reported the bicyclic pyrrolo[2,3-\textit{d}]pyrimidine compound \textit{161}, with the \textit{N}-methyl-4’-methoxyaniline moiety at the 4-position, as a potent microtubule depolymerizing agent (Figure 47).

**Table 8.** Disruptive effect on microtubule polymerization in A-10 cells, and inhibition of proliferation of MDA-MB-435 cells for \textit{161}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC\textsubscript{50} (nM) in A-10 cells</th>
<th>IC\textsubscript{50} (nM) (MDA-MB-435)</th>
<th>Inhibition of colchicine binding (% Inhibition ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{161}</td>
<td>5.8 µM</td>
<td>183 nM</td>
<td>70 %</td>
</tr>
</tbody>
</table>

In the preclinical screening program of the National Cancer Institute 60-cancer cell line panel, \textit{161} was found to inhibit the proliferation of most of the cancer cell lines with a GI\textsubscript{50} of less than 500 nM. It also caused a dramatic reorganization of the interphase microtubule network, which was similar to the effects of CA4-P (Table 7).\textsuperscript{228}

Unlike paclitaxel, it maintained most of its potency against P-gp-overexpressing cell lines and showed slightly better activities against β-III-tubulin-overexpressing cancer cell lines.\textsuperscript{228} Moreover, it displaced 70\% of the [\textsuperscript{3}H] colchicine at a concentration of 5 µM. Thus, it was determined to be a colchicine binding agent. Prior to this work,\textsuperscript{228} there were sparse reports on pyrrolo[2,3-\textit{d}]pyrimidine-based colchicine-site binding agents. This discovery was an inspiration to explore the SAR of this class of compounds as MTAs.

Based on the above data, \textit{161} was an excellent lead compound for further development and **Series IX** (Figure 47) addresses the effect of substituting the \textit{N}-methyl-4’-methoxyaniline moiety with various anilines in the 4-position of the pyrrolo[2,3-\textit{d}]pyrimidine ring of \textit{161}.  

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Compound 162 (Figure 47), a conformationally restricted aniline with a fused bicyclic 4-N-methyl-2'-3'-dihydrobenzofuran moiety at the 4-position of 2-methyl-7H-pyrrolo[2,3-d]pyrimidine was designed. Bond rotation about rotatable single bond, b in the lead molecule 161 was restricted in compound 162, thus, affording a much more rigid structure than 161 but still maintaining the phenyl and alkyl substitutions on the N4 as in 161. Conformational restriction of the flexible 4-anilino moiety would minimize the entropic loss associated with the 4-anilinio moiety adopting a preferred conformation for binding to tubulin. This could enhance potency for tubulin. Another advantage could be reduced drug metabolism. The electron poor 4’-methoxypyridine moiety was designed in place of the 4’-methoxyaniline moiety of 161 in 163. Napthols 164 and 165 would determine if the 4’-methoxy and 4N-methyl are necessary for activity. It was hypothesized that fusion of another aromatic ring at the appropriate positions of the pyrrolo[2,3-d]pyrimidine 161 would improve tubulin inhibitory potency. Compound 166 with a 5’-methoxy-N-methyl-2-naphthalenamine moiety would determine if additional bulk would lead to increase in biological activity. Compound 167 was designed by replacing the 4’-methoxy of the lead 161 with a 4’-thiomethyl moiety as discussed earlier in Figure 40.

Rationale explained further: As phenyl ring of 161 can exist in multiple conformations this can result in unexpected entropic costs while binding to the colchicine bind site of tubulin. High entropic cost would offset any possible enthalpic gains that may have been achieved while binding to the active site. Hence, compounds 162 were designed as conformationally restricted analog of 161 restricting the rotation about bond b (Fig. 47, pg 94). A series of restricted analogs have already been designed and synthesized for other scaffolds like
thieno[2,3-\textit{d}]pyrimidines and pyrrolo[2,3-\textit{d}]pyrimidines, and it is found that the replacement of 4'-methoxyaniline moiety with bicyclic 6-methoxy-tetrahydroquinoline and 4-N-methyl-2'-3'-dihydrobenzofuran moiety are the most active as they maintain the phenyl and alkyl substitution at N4 of 161. 6-methoxy-tetrahydroquinoline replacement has already been done by Dr. Lin in our group. Also, the 4-OR of 162 maintained the hydrogen bonding interaction with Cys241 in tubulin binding site.

A series of compounds with electron withdrawing groups eg halogens, OCF$_3$ at para position were synthesized by Dr. Lin. Compound 163 with an electron poor 4'-methoxypyridine moiety was designed to determine if an electron poor ring would be beneficial for the inhibitory potency. 4'-methoxypyrimidine moiety has already been synthesized by Dr. Lin.

Nitrogen containing heteroaromatic rings are ubiquitously present in drug-like molecules and 59% of U.S. FDA approved small-molecule pharmaceuticals contain at least one nitrogen heterocycle. Substituting a CH group with a N atom (as in 163) can lead to improvements in functional activities such as biochemical potency, cellular potency, and target selectivity. It redistributes electron density in the ring and introduces dipole moment ($\mu=2.2$ D), hydrogen bond capability, and polarity into the molecule. Other students in our group will be performing a systematic N atom scan (N-scan) by exchanging the trivalent CH groups in the phenyl side chain of the ligand 161 with trivalent N atoms, one at a time, to determine its optimal placement for desired pharmacological improvements.
A series of compounds replacing phenyl with substituted naphthalenyl moiety has already been synthesized by Dr. Lin in our group. Compounds 164-166 were designed to determine the bulk tolerance in the colchicine site of tubulin. The increased bulk can form hydrophobic interactions with hydrophobic residues Ala250, Leu248, Ile1042 and Val914.

The 4N-Me analog of 164 has already been synthesized by Dr. Lin. To determine the role of N-Me in hydrophobic interactions and to also determine if N-H would be involved in any hydrogen bonding interactions as a donor, 164 was designed.

The 4N-Me and 4’O-Me analog of 165 has been synthesized by Dr. Lin. To determine if these methyl groups are conducive to microtubule depolymerization, 165 with 4NH and 4’OH were designed. These extra H groups can also lead to improved activity because of extra hydrogen bonding interactions with the target as hydrogen bonding donors.

Compound 166 with a 5’-methoxy-N-methyl-2-naphthalenamine moiety was designed because of the improved biological results in other scaffolds when 4’-methoxyaniline moiety was replaced with a 5’-methoxy-N-methyl-2-naphthalenamine moiety. It was found that position of 5’-methoxy group in the naphthalene ring plays a big role in the biological activity against tubulin polymerization. 5’-methoxy group maintains the hydrogen bonding interaction with Cys241.

The replacement of 4’-OMe with 4’S-Me in 167 was done to determine the bulk tolerance (O vs S) as well as the importance of hydrogen bonding. Sulfur being a bulkier hydrophobic
group could increase the activity by increasing the hydrophobic interactions at the colchicine site of tubulin.

The S-Me compound 167 was also designed because it showed improved activity in other scaffolds. For instance, when the 4’-methoxy group of 300 (Fig. 40) was replaced with a 4’-thiomethyl group (301), it led to a 2-fold increase in activity against tubulin, and a 1.5-fold increase in inhibition of cell proliferation.

**Oxazolo[5,4-d]pyrimidines as microtubule targeting agents**

![Series X](image)

**Figure 48.** Design rationale for Series X

Compounds 168 and 169 (Figure 48) were recently reported\(^{82-83,180}\) by Gangjee et al. as potent MTAs that bind in the colchicine site of tubulin. Compounds 170-171 were designed as hybrids of these molecules to determine the effect of oxazolo compounds on tubule polymerization.
Pyrimido[4,5-g]pteridine as a potential MTA.

Figure 49. Design rationale for dimer 172

A number of tricyclics \(^{183, 229-230}\) have been reported by Gangjee et al. which act as MTAs. The dimer 172 (Figure 49) was obtained via self-condensation of 122, and it was of interest to evaluate its effects on tubulin polymerization.

Selective tumor targeting with 5-substituted pyrrolo[2,3-d]pyrimidines with heteroatom bridge substitution

The lack of tumor selectivity for all the currently clinically used antifolates, including methotrexate (MTX), pemetrexed (PMX), pralatrexate (PDX), and raltitrexed (RTX), reflects their cellular uptake by the reduced folate carrier (RFC) expressed ubiquitously by both normal tissues as well as tumors.\(^{231}\) In contrast, folate receptor (FR) \(\alpha\) and \(\beta\), and the proton-coupled folate transporter (PCFT) exhibit selective tumor tissue expression, and serve specialized physiological roles.\(^{113}\) FRs on normal cells are inaccessible to circulating folates or are non-functional in normal tissues. PCFT is expressed in the upper gastrointestinal tract where it functions only at acidic pH as the major intestinal transporter.
for absorption of dietary folates. Given this requirement of acidic pH for activity, PCFT transport is limited in normal tissues that are not at acidic pH (<6.9). A substantial cohort of solid tumors (e.g., ovarian, non-small cell lung cancer) express PCFT, often in concert with FRs. FRα is accessible on tumors, thus, allowing tumor targeting by this mechanism. FRβ is expressed in hematological malignancies and in white blood cells of the myeloid lineage, including tumor-associated macrophages (TAMs). FRβ-positive TAMs may play an important role in the tumor microenvironment in reaction to tumor metastasis and angiogenesis by releasing proangiogenic factors suggesting that TAMs may constitute an additional potential therapeutic target in cancer for FRβ-targeted agents. There are no tumor targeted cytotoxic agents in clinical use, thus, all cytotoxic agents display dose-limiting toxicity. New cytotoxic agents are needed which can target tumor cells selectively by virtue of their specificities for FRs and/or PCFT and thus, afford agents that do not suffer from dose-limiting toxicities.
PMX is currently among the most prescribed antitumor agents used in the clinic.\textsuperscript{232} The principal indications for PMX are malignant pleural mesothelioma (with cisplatin) and non-small cell lung cancer. Neutropenia is the major dose-limiting toxicity associated with PMX, and reflects in part, its nonselective uptake into normal cells, via RFC, expressed in normal as well as tumor cells.\textsuperscript{233} Gangjee et al.\textsuperscript{234} previously reported a 5-substituted pyrrolo[2,3-$d$]pyrimidine antifolate with a 5-carbon bridge and a phenyl $L$-glutamate side chain, \textbf{302} (Figure 50). These compounds were part of a series of chain extension analogs of clinically used PMX to improve tumor cell potency as well as specificities for FRs and/or PCFT. Although, \textbf{302} was found to be 3.5-fold more potent in KB tumor cells than clinically used PMX, and showed potent inhibition of CHO cells expressing FR$\alpha$ (IC$_{50}$ = 33.5 nM), it had reduced activity toward CHO cells expressing PCFT, and high activity toward CHO cells expressing RFC, thus, it lacked selectivity for FR and/or PCFT. Previous studies of related 6-substituted pyrrolo[2,3-$d$]pyrimidines established that the nature and length of the bridge plays an important role in determining tumor cell potency and selectivity.\textsuperscript{124} Thus, it was of interest to insert heteroatom into the carbon bridge of \textbf{302} to determine if O or S substitution of C would afford better activity. Compounds \textbf{173-176} (Figure 50) were designed and their impact on drug potency and transport selectivity was determined. The novel analogs were tested as growth inhibitors against CHO cells expressing human FR$\alpha$ (RT16), FR$\beta$ (D4), RFC (PC43-10), and PCFT (R2/PCFT4) as well as KB tumor cells in culture.
Molecular Modeling Studies

Figure 51. Superimposition of the docked poses of 174 (grey) and PMX (green) in the folate binding site of human FRα (5IZQ). Compounds 173-176 and PMX were docked in the folate binding cleft of FRα (PDB 5IZQ) using MOE2013.0801 software to predict potential binding of these analogs for uptake by FRα. Figure 51 shows the superimposition of docked poses of 174 and PMX in the human FRα active site. Compound 174 displayed similar interactions in the pocket, maintaining important protein contacts between the bicyclic scaffolds and benzoyl L-glutamate tail, as also seen in the crystal structure ligand 6-substituted pyrrolo[2,3-\textit{d}]pyrimidine (not shown) and docked standard compound PMX.
Figure 52. Superimposition of the docked poses of 173 (green) and 6-substituted pyrrolo[2,3- d]pyrimidine 304 (grey) in the 10-formyl tetrahydrofolate binding site of GARFTase (4ZZ1).

In the docked pose of 174 (Figure 51), the 2-hydroxy of 174 interacts with Ser174. The 4-oxo moiety forms hydrogen bonds with the side chain NH of Arg103 and the side chain OH of Ser174. The pyrrolo[2,3- d]pyrimidine scaffold is stacked between the side chains of Tyr60 and Trp171. The L-glutamate moiety of 174 is oriented similar to the corresponding L-glutamate in PMX. The γ-carboxylic acid of 174 interacts with the
protonated amine of Lys136 and the side chain OH of Ser101. The bridge of 174 forms hydrophobic interactions with Tyr60, Phe62, Trp102, and His135. The docking score of 174 was −13.29 kJ/mol (rmsd = 1.68), somewhat better than the docked scores of PMX (−12.36 kJ/mol, rmsd = 1.68), and suggests better or similar activity for 174 at FRα.

Figure 52 shows docked 173 in the crystal structure of human GARFTase (PDB 4ZZ1) complexed with 6-substituted pyrrolo[2,3-d]pyrimidine 304. Compounds 173-176 were docked into the X-ray crystal structure of human GARFTase to predict potential activities for GARFTase inhibition. The pyrrolo[2,3-d]pyrimidine scaffold of 173 binds in the region occupied by the bicyclic scaffold of the bound molecule 304 in GARFTase. Hydrogen bonds between the N1 nitrogen of 173 and the backbone of Leu899; 2-NH2 of 173 and the carbonyls of Glu948 and Leu899, N3 of 173 and the backbone C=O of Ala947, and 4-oxo of 173 and the backbone C=O of Asp951 stabilize the scaffold. The pyrrolo[2,3-d]pyrimidine scaffold of 173 forms hydrophobic interactions with Leu892, Ile898 and Leu899. The docking score of 173 was −14.18 kJ/mol. Similarly, compounds 174-176 had similar docked scores indicating potent GARFTase inhibition. Thus, the molecular modeling studies provided support for the synthesis and biological evaluation of 173-176.
IV. CHEMICAL DISCUSSION

Figure 53. Retrosynthesis of target compound 130

Synthesis of 130 was envisioned to be accomplished via a simple Suzuki coupling as benzyl boronic esters/acids are easily available (Figure 53). The intermediate 122 could be synthesized via nucleophilic substitution reaction\textsuperscript{229} of 177 with N-methyl 4-methoxy aniline.

Scheme 26. Formation of Meisenheimer complex\textsuperscript{235}
**Synthesis of 122 and 156**

With the above synthetic strategy for the synthesis of 130, key intermediate 122 was synthesized by treating 177 with 1.1 equivalents of N-methyl-4-methoxyaniline and catalytic amounts of hydrochloric acid. The reaction was also attempted without addition of any HCl but leads to 25-30% less yields. Reactions with catalytic amounts of HCl provided better yields as HCl protonates the pyridine nitrogen next to the C-Cl bond (Scheme 26).

![Scheme 27. Synthesis of 156 and 122](image)

Reagents and conditions: N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 150 °C, MW, dioxane

The reactions were attempted in a microwave reactor at 150 °C as described in the literature. However, it was found that higher temperature afforded a disubstituted product 156, and significantly reduced the yield (15 % product formation, Scheme 27).
### Table 9. Effect of variation in temperature

<table>
<thead>
<tr>
<th>Equiv of Nucleophile used</th>
<th>Temperature</th>
<th>Major product formed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>150 °C</td>
<td>Disubstituted product</td>
</tr>
<tr>
<td>1.1</td>
<td>120 °C</td>
<td>Monosubstituted product</td>
</tr>
</tbody>
</table>

### Scheme 28. Synthesis of 122

![Chemical structure](image)

Reagents and conditions: N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane

Lower temperatures (Table 8) were then used, and the reaction temperature was optimized to significantly decrease formation of 156, affording 122 in 75% yield (Scheme 28).

A $^1$H NMR study was also done in DMSO-d$_6$ to determine the conformation of 122. According to the $^1$H NMR spectrum, the 5-NH$_2$ protons of 122 are more shielded and come at 4.0 ppm compared to the 5-NH$_2$ protons of starting material 177 which come at 5.92 ppm. This suggested a nearby diamagnetic anisotropic cone. Due to the bulk of the 4N-Me group, the conformation of 122 is restricted, and the phenyl ring of 122 positions itself on the top of the 5-NH$_2$ protons (as shown in Scheme 28) leading to the observed shielding...
effect.\textsuperscript{83} It was observed that lower temperature, less reaction time and less equivalents should be used to produce monosubstituted reactions.

**Synthesis of 130**

Compound 122 was subjected to a Suzuki coupling using potassium benzyltrifluoroborate to afford 130.\textsuperscript{238} The reaction was performed in a microwave for 5-10 min with palladium triphenylphosphine as the catalyst and cesium carbonate as the base to afford benzyl product 130. The reaction was carefully monitored by thin layer chromatography (TLC). A new product (red spot) was observed on TLC with other side products, and after extracting the product with ethyl acetate and drying under sodium sulfate, column chromatography afforded the product. Purification of 130 required extensive column chromatography. Variations in temperature, time, catalyst, solvent and boronic reagents were carried to afford a pure product (Scheme 29).

**Scheme 29.** Variations in temperature, time, catalyst, solvent and boronic reagents

![Scheme 29](image)

Reagents and conditions: Pd catalyst, Cs\textsubscript{2}CO\textsubscript{3}, MeCN : Water = 1 : 1, MW
Table 10. Variations in temperature, time, catalyst, solvent and boronic reagents

<table>
<thead>
<tr>
<th>No.</th>
<th>Boronic cmpds</th>
<th>Temp./Time</th>
<th>Solvents</th>
<th>Pd cat.</th>
<th>Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>150 °C / 5min</td>
<td>Dioxane/H₂O</td>
<td>Pd(PPh₃)₄</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>150 °C / 30 min</td>
<td>Dioxane/H₂O</td>
<td>Pd(PPh₃)₄</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>150 °C / 30 min</td>
<td>Dioxane/H₂O</td>
<td>Pddppf</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>80 °C / 30 min</td>
<td>THF/H₂O</td>
<td>Pddppf</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>120 °C / 1 h</td>
<td>Acetonitrile/H₂O</td>
<td>Pd(PPh₃)₄</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>120 °C / 1 h</td>
<td>Acetonitrile/H₂O</td>
<td>Pd(PPh₃)₄</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>120 °C / 1 h</td>
<td>Acetonitrile/H₂O</td>
<td>Pd(PPh₃)₄</td>
<td>Yes</td>
</tr>
</tbody>
</table>

After the pure product was isolated using conditions mentioned in entry 7 (Scheme 29). ¹H NMR analaysis of the isolated product showed the absence of any amino protons at the 5-position of 130. ¹H NMR analysis also showed the absence of benzyl methylene protons or the phenyl protons.
Figure 54. $^1$H NMR of Suzuki coupling product which yielded 172 instead of 130.

Compound 178 was synthesized to determine whether its $R_f$ was similar to the final product but was found to be different, and so, one possibility was the formation of a dimer product 172 (Scheme 30).
The pure isolated product was further analyzed for CHN and mass spectrometry which proved the formation of a dimer product 172. The mass of 122 was 270.09 while the mass of the dimer 172 was 482.55. The % of element C in the isolated product was found to be 64.5% which confirmed that 172 was formed. If it were 122, the % of element C would have been 56.3%.
Scheme 31 Synthesis of 172

Reagents and conditions: a. Pd(PPh₃)₄, Cs₂CO₃, MeCN: Water = 1 : 1, 120 °C, 30 min, MW  
b. Pd(PPh₃)₄, MeCN: Water = 1 : 1, Cs₂CO₃, MW, 120 °C, 30 min

Scheme 32. Synthesis of 172 without palladium catalyst and boronic reagent was unsuccessful

Reagents and conditions: acetonitrile/H₂O, Cs₂CO₃, MW, 120 °C, 30 min.
To evaluate 172 in biological assays, it was resynthesized. It was observed that the reaction fails without addition of any palladium catalyst and/or boronic reagent (Schemes 31-32). Interestingly, it was found that the boronic ester/salt is required for the formation of the dimer.

Reason Suzuki coupling might have failed: Although the actual mechanism is still unresolved, the boronic reagent and palladium could be involved in the oxidative addition and transmetallation steps of the Suzuki coupling. But before reductive elimination occurs to form 130, the 5-amino could attack and form the dimer 172.

**Scheme 33.** Failure of the protection of the 5-amino using pivalic anhydride.

![Scheme 33](attachment:image.png)

Reagents and conditions: Pivalic anhydride, 130 °C, 12 h

The 5-amino of 122 was then protected using pivalic anhydride to prevent dimer formation but the protection failed to afford 135 (Scheme 33).
Figure 55. $^1$H NMR of pivalic protection isolated product yielded 171 instead of 135.

After the pure product was isolated (observed on TLC), $^1$H NMRs of isolated product showed absence of 5-NH. One possibility was formation of a bicyclic oxazolo[5,4-$d$]pyrimidino compound 171 (Scheme 34). Elemental (CHN) analysis and mass spectrometry confirmed the presence of the oxazole (Figure 55). The mass of product formed was 326.17, which is the correct mass of 171 (with loss of one Cl and one H), while the mass of the 135 is 362.15. The % of element C was found to be 66% which confirmed the formation of 171.
Scheme 34. Synthesis of 171

Reagents and conditions: Pivalic anhydride, 130 °C, 12 h

Table 11. Effect of variation in temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 °C</td>
<td>N-pivyl formation</td>
</tr>
<tr>
<td>150 °C</td>
<td>oxazolo[5,4-d]pyrimidinyl formation</td>
</tr>
</tbody>
</table>

Lower temperatures were then used, and the reaction temperature was optimized to significantly decrease the formation of 171, and to afford 135 so that the target benzyl compound 130 could be synthesized (Table 9).

It was observed that low temperatures lead to pivalyl protection (78% yield), and higher temperatures lead to bicyclic oxazolo formation (60% yield).

**Synthesis of 133**

The reaction of 122 with acetic anhydride will serve two purposes:

1. Compound 170 (Scheme 35) could be synthesized at higher temperatures which could function as a potent microtubule targeting agent. This would afford novel
oxazolo[5,4-\(d\)]pyrimidinyl compounds that have not been reported as MTAs in the literature.

2. At lower temperature, acetyl protection could be achieved which would prevent dimer formation under Suzuki conditions.

Scheme 35. Synthesis of 133 and 134.

Reagents and conditions  
a. Acetic anhydride, 100 °C, overnight  
b. Acetic anhydride, 150 °C, overnight

The 5-amino group in 122 was reacted with acetic anhydride at both lower and higher temperatures (Scheme 35). Very interesting results were obtained.

Low temperatures, without basic conditions, afforded the N-acetyl product 133. Higher temperatures without any base afforded N-acetyl-acetamide 134 instead of 170 (Table 10).
Table 12. Effect of variation in temperature.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Temperature</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>No base</td>
<td>100 ºC</td>
<td>N-acetyl formation</td>
</tr>
<tr>
<td>No base</td>
<td>150 ºC</td>
<td>N-acetyl-acetamide formation</td>
</tr>
<tr>
<td>Base, CuI</td>
<td>110 ºC</td>
<td>Oxazole formation</td>
</tr>
</tbody>
</table>

Scheme 36. Synthesis of 170

![Scheme 36](image)

Reagents and conditions: CuI, DMEDA, K$_2$CO$_3$, Toluene, reflux, 6 h, 55% yield

Finally, the synthetic method by Xu et al.$^{239-240}$ which reported intramolecular C-O cross-coupling of the o-halogenopyrimidine amide 133 via Cu-mediated cyclization afforded exclusively the oxazolo[5,4-\(d\)]pyrimidino 170 in 55 % yield (Scheme 36).
Synthesis of 150

Scheme 37. Attempted Suzuki coupling with 133 and 135.

Reagents and conditions: a) acetonitrile/H$_2$O, Cs$_2$CO$_3$, Pd(PPh$_3$)$_4$, MW, 120 °C, 30 min

Acetylated (133) (Scheme 37) and pivylated (135) products were subjected to Suzuki coupling to afford 130. Unfortunately, the reactions under Suzuki conditions did not work.

Reasons for the failure of the Suzuki reaction with 133 and 135.

1. The bulk of the acetyl and pivyl groups could make the attack of the boronic salt difficult during the transmetallation step of the Suzuki coupling.

2. Chlorides are not good leaving groups for coupling reactions like the Suzuki. Better groups like bromides or iodides are necessary.$^{241-242}$
Base plays a major role in the transmetallation step of the Suzuki coupling.\textsuperscript{242} The presence of a base might also be the main reason for the formation of dimers in Suzuki coupling as it makes the 5-amino a better nucleophile (Scheme 31). Hence, Negishii coupling was attempted on 122 as this coupling does not require base and could prevent dimer formation (Scheme 38).

**Scheme 38.** Attempted Negishii coupling with 122 was unsuccessful.

![Scheme 38](image.png)

Reagents and conditions  Pd(PPh\textsubscript{3})\textsubscript{4}, THF, 60 °C, 2 h (inert)

Compound 122 was treated with 0.5 M solution of benzyl zinc bromide in THF under Negishii conditions to yield 133.\textsuperscript{243} The reaction was carried out under inert conditions. There was no sign of any product or side product formation. No base was used, and that could be the reason for failure of products even dimer formation. The absence of a good leaving group like a bromide/iodide might be the reason behind the failure of the Negishii reaction.
Scheme 39. Synthesis of 142 with sodium iodide was unsuccessful.

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane b) NaI, TMS-Cl, MeCN, 180 °C, MW

Synthesis of 142 - Iodination

The next step was to introduce an iodo moiety at the 6-position. Yang et al.\textsuperscript{134} and Lemoine et al.\textsuperscript{135} introduced the iodo by reacting chloride with NaI and TMS-Cl in acetonitrile at 180 °C in a microwave reactor. The same method was followed but it failed for the pyrimidine scaffold (Scheme 39).

Scheme 40. Synthesis of 142

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 75 % yield b) 57 % HI (aq.), MeCN, 0 °C- rt, 48% yield
Finally, the method used by Hughes et al.\textsuperscript{137} worked successfully for the substitution of the chloro group with an iodo group with 57% aqueous hydroiodic acid in acetonitrile (Scheme 40). Compound 122 was treated with a 57% aqueous solution of hydroiodic acid at 0 °C to afford 142 in 48% yield.

Replacement of the chloro moiety with an iodo was a crucial step for the final synthesis of the target 6-benzyl compound 130.

**Synthesis of 130**

Scheme 41. Synthesis of 130

![Scheme 41](image)

Reagents and conditions: Pd(PPh\textsubscript{3})\textsubscript{4}, THF, 60 °C, 2 h (inert), 35% yield

Compound 142 (Scheme 41) was treated with 0.5 M solution of benzylzinc bromide in THF under Negishi conditions to afford 130 in 35% yield. The reaction was carried out under inert conditions.

Why Negishi coupling might have worked:
1. The use of zinc metal could help in forming a coordination complex with nitrogen of pyrimidine leading to reduction in electrophilicity of C-Cl, and facilitating coupling reaction. This coordination was not possible with Suzuki coupling.

2. No base was used, and that must be the reason for the lack of formation of any side products like dimer formation.

**Synthesis of 151**

**Scheme 42.** Attempted synthesis of 131 under Suzuki conditions.

Reagents and conditions: acetonitrile/H$_2$O, Cs$_2$CO$_3$, Pd(PPh$_3$)$_4$, MW, 120 °C, 30 min

**Suzuki coupling for phenylethyl product formation**

Attempted syntheses of 131 under Suzuki conditions (Scheme 42) were unsuccessful.
**Scheme 43.** Attempted Negishi coupling for the synthesis of 131.

![Chemical structures](image)

Reagents and conditions: Pd(PPh₃)₄, N₂, 60 °C, THF

**Negishi coupling for phenylethyl product 131 formation.**

Although the treatment of 142 with 0.5 M solution of phenylethyl zinc bromide and palladium catalyst worked, it led to side product formation which could not be isolated using column chromatography (Scheme 43).

As both Suzuki and Negishi coupling reactions failed to afford 131, Sonogoshira coupling was attempted.
**Scheme 44.** Attempted Sonogoshira reaction with the chloropyrimidine 122.

\[
\begin{align*}
\text{122} & \quad \text{H} = \text{-} \quad \text{132} \\
\end{align*}
\]

Reagents and conditions: CuI, PdCl\(_2\)(PPh\(_3\))\(_2\), NEt\(_3\), dry DCE, 100 °C, 2 h

**Sonogoshira reaction for phenylethyl product formation.**

The Sonogoshira reaction was not successful with the chloropyrimidine (Scheme 44). However, the iodide 142 did afford 131 under the same conditions.

**Scheme 45.** Synthesis of 131

\[
\begin{align*}
\text{142} & \quad \text{H} = \text{-} \quad \text{131} \\
\end{align*}
\]

Reagents and conditions: a) CuI, PdCl\(_2\)(PPh\(_3\))\(_2\), NEt\(_3\), dry DCE, 100 °C, 2 h, 65 % yield b) H\(_2\) (50 psi), 10 % Pd/C, rt, 35 % yield.
Compound 142 was coupled with phenylacetylene under Sonogoshira coupling conditions to afford 132 in 65% yield which was then catalytically hydrogenated at 50 psi using 10% Pd/C to yield 131 (Scheme 45).

**Synthesis of 154-159**

**Scheme 46. Synthesis of 123, 125-128**

Reagents and conditions : a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 75 % yield b) NaH, alkyl halide, DMF, 2 h, 0° C-rt, 35- 78% yields c) Diethyl ether, HCl (g), 90 % yield.

2-Methyl-4,6-dichloropyrimidine 177 (Scheme 46) was subjected to nucleophilic aromatic substitution using N-methyl-4-methoxyaniline and catalytic amount of conc. HCl in
dioxane to afford 122. Reactions involving microwave irradiation afforded 15-25% better yields than conventional bench-top conditions. Compound 122 was deprotonated with sodium hydride, and treated with the appropriate alkyl halide to afford the corresponding N5-alkylated compounds 123-128 in 35-78 % yields. Reactions involving combinations of cesium carbonate or potassium carbonate and dimethylformamide with microwave irradiation did not show complete consumption of starting material. The use of excess base or alkyl halide did not afford the alkylated product exclusively. To increase water solubility, the products were converted to the HCl salt using HCl gas and ether. The composition of element chloro was found to be 19.15% for 127·HCl (C_{17}H_{23}ClN_{4}O·1.0HCl) which confirmed the formation of the HCl salt.

**Synthesis of 124**

Scheme 47. Synthesis of 124

Reagents and conditions: NaH, MeI, DMF, 2 h, 0º C- rt

<table>
<thead>
<tr>
<th>Base equiv.</th>
<th>MeI equiv.</th>
<th>Ratio (% yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0.9</td>
<td>123:124 = 70:30</td>
</tr>
</tbody>
</table>
Deprotonation of 122 with 0.9 equiv. of sodium hydride, and treatment with 0.9 equiv. of MeI afforded a mixture of yield of (70%) 123 and 30% of the N5-dialkylated 124 (Scheme 47). The products could be easily separated using column chromatography due to different RfS on TLC (Rf = 0.62 for 123 and 0.65 for 124). CHN elemental analysis confirmed the formation and separation of both products with C = 58.06%, H = 5.97%, N = 18.76%, Cl = 11.85% for 123 and C = 59.16%, H = 6.37%, N = 17.71%, Cl = 11.23% for 124. $^1$H NMR analysis for 124 showed a sharp peak at 2.14 ppm which integrated for 6 protons while for 123, the sharp peak at 2.26 ppm integrated for 3 protons only. Increasing equivalents of base and alkylating agent from 0.9 to 2 equiv. increased yield of 124 to 50% of the mixture.

### Synthesis of 136

**Scheme 48.** Synthesis of 136

![Synthesis of 136](image)

Reagents and conditions: N-methyl-4-methoxyaniline, cat. HCl (conc.), 3 h, 160 °C, MW, dioxane, 82% yield.

2-Nitro-4,6-dichloropyrimidine 178 (Scheme 48) was subjected to a nucleophilic aromatic substitution reaction using 4-methoxy aniline and catalytic amount of conc. HCl in dioxane.
to afford 136 in 82% yield. The higher yield was obtained due to the presence of a strong electron withdrawing nitro group which makes the 4-position of 178 highly electrophilic.

**Synthesis of 137**

2-Methyl-4,6-dichloropyrimidine 177 (Scheme 49) was subjected to a nucleophilic aromatic substitution using 4-methoxy aniline and catalytic amount of conc. HCl in dioxane to afford 122. The use of Pd/C as for hydrogenation afforded 137 in 65 % yield.

2-Methyl-4,6-dichloropyrimidine 177 (Scheme 49) was subjected to a nucleophilic aromatic substitution using 4-methoxy aniline and catalytic amount of conc. HCl in dioxane to afford 122. The use of Pd/C as for hydrogenation afforded 137 in 65 % yield.

**Scheme 49. Synthesis of 137**

![Scheme 49. Synthesis of 137](image)

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 75 % yield  b) H₂ (50 psi), 10 % Pd/C, rt, 65 % yield.
**Synthesis of 138-139**

**Scheme 50. Synthesis of 138-139**

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane
b) Al(Me)$_3$, Pd(PPh$_3$)$_4$, THF, 90 °C, 2 h, 65 % yield
c) N-methyl 4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane
d) Al(Et)$_3$, Pd(PPh$_3$)$_4$, THF, 90 °C, 2 h, 58 % yield.

2-Methyl-4,6-dichloropyrimidine 177 (Scheme 50) was subjected to a nucleophilic aromatic substitution reaction using 4-methoxyaniline and catalytic amount of conc. HCl in dioxane to afford 122 which was then coupled with the appropriate trialkyl aluminium
reagent using palladium catalyst to afford 138 and 139 in 65% and 58% yields respectively.

**Synthesis of 140**

**Table 13.** Maximum temperature afforded with different types of ammonia solution.

<table>
<thead>
<tr>
<th>Ammonia type used</th>
<th>Max. Temperature attained in microwave (MW)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous (35 %)</td>
<td>120 °C</td>
<td>No product formed</td>
</tr>
<tr>
<td>Ammonia in MeOH (7 N)</td>
<td>140 °C</td>
<td>10%</td>
</tr>
<tr>
<td>Ammonia in EtOH (2 N)</td>
<td>160 °C</td>
<td>65%</td>
</tr>
</tbody>
</table>

**Scheme 51. Synthesis of 140**

![Scheme 51. Synthesis of 140](image)
Reagents and conditions: a) NH$_3$ in ethanol solution, 160 °C, 3 h, MW, 42% yield b) N-methyl N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 75% yield.

For the amination of 177 (Scheme 51) at the 6-position, it was treated with aqueous ammonia solution at 100 °C to afford 179 (Table 11). The reaction was unsuccessful, and so ammonia in methanol and ethanol were attempted to achieve higher reaction temperatures. Ammonia in ethanol afforded microwave temperatures 20° to 40° higher and also afforded better yields compared to ammonia in methanol (Table 11). Compound 179 (Scheme 51) was subjected to a nucleophilic aromatic substitution using 4-methoxyaniline and catalytic amount of conc. HCl in dioxane to afford 140. However, this reaction failed.

Why reaction may have failed: The displacement of the 6-Cl of 177 with an amino group makes the 4C-Cl of 179 less electrophilic which could be the reason of the failure of the displacement reaction. The reverse sequence of addition was attempted which worked successfully to afford 140. First, displacement with aniline was carried out to afford 122 in 75% yield, followed by amination with a solution of ammonia in ethanol to afford 140 in 42% yield.
**Synthesis of 141**

**Diazonium salt formation**

**Scheme 52. Synthesis of 181**

Reagents and conditions: a) Pivalic anhydride, 120 °C, 3 h, 65 % yield b) NH₃ in ethanol solution, 160 °C, 3 h, MW, 42 % yield c) i. NaNO₂, H₂O, HCl, 0 °C ii. CuCN, H₂O, 60 °C, 15 min.

Compound 135 (Scheme 52) was treated with an ethanol solution of ammonia to yield 180 which in turn was converted to a diazonium salt using sodium nitrite under acidic conditions, and then converted to the nitrile using copper cyanide. The use of acid in this reaction led to the cleavage of the methoxy group into the phenol (182 formation).
Fusion method

**Scheme 53.** Attempted synthesis of 141 using fusion method

Reagents and conditions: a) N-methyl 4-methoxy aniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane  
 b) CuCN, fusion, 150 °C, 2 h

The failure of the previous synthesis of 141 prompted Fusion method in which the reaction of 122 and copper cyanide was performed in a sealed microwave vial at 150 °C without the use of any solvent but it too failed to afford 141 (Schemes 53).\(^{138}\)

Why the reaction may have failed: The lack of solvent might be the reason behind failure of the reaction, as optimum collision between molecules is required to cause bond formation.

**Scheme 54.** Attempted synthesis of 141 using CuCN-DMSO mixture.

Reagents and conditions: CuCN, DMSO, argon, 100 °C, 12
Next, DMSO was added as a solvent to determine the effect of addition of a high boiling solvent, however this too failed to afford 141 (Scheme 54).

**Scheme 55.** Attempted synthesis of 141 using DABCO as base

![Chemical structure of 122 and 141](image)

Reagents and conditions : CuCN, DABCO, DMSO : H₂O, 9:1, 65 °C, 30 h

Another reaction with DMSO-water mixture, and DABCO as base was attempted to afford 141 but was also unsuccessful (Scheme 55).

*Rosenmund-von Braun reaction* \(^{137}\)

**Scheme 56.** Attempted synthesis of 141 using Rosenmund-von Braun reaction.

![Chemical structure of 122 and 141](image)

Reagents and conditions : CuCN, DMF, 180 °C, 12 h
Next, the Rosenmund-von Braun reaction with copper cyanide in a polar solvent like DMF and NMM at higher temperatures (180 °C) was attempted but also failed to yield the desired nitrile product 141 (Scheme 56).\(^{137}\)

Why the reaction may have failed: The 6-Cl is not electrophilic enough for reaction to occur. The high temperature and longer hours did not work successfully here.

Another reason: Rosenmund-von Braun reaction works better with iodides rather than chlorides, so next iodides should be attempted.

**Scheme 57. Synthesis of 141**

![Diagram of reaction](attachment:image.png)

Reagents and conditions : CuCN, DMF, 180 °C, 12 h, 22 % yield.

Another high boiling solvent, NMP, was used instead of DMF but also failed to afford 141. The Rosenmund-von Braun reaction works better with iodides rather than chlorides,\(^{137}\) and so, 142 (Scheme 57) with a 6-I was treated with copper cyanide which in a high boiling solvent like DMF finally worked successfully with complete disappearance of the starting material on TLC affording the desired nitrile 141.
It was found that for the substitution reactions, it always better to do reactions on iodides or bromides than chlorides as iodides/bromides are better leaving groups. Chlorides are better for $S_{N}Ar$ kind of reactions, where electrophilicity plays more important role.

**Scheme 58.** Attempted variation of the classic Sandmeyer reaction.

![Scheme 58](image)

Reagents and conditions: a) Pivalic anhydride, 120 °C, 3 h b) NH$_3$ in ethanol solution, 160 °C, 3 h, MW c) $t$-BuNO$_2$, ACN, HCl, 0 °C, 15 min. d) AgCF$_3$, -78 °C, 3 h

**Synthesis of 143- Trifluoromethylation reaction.**

Wang et al. used a novel strategy for aromatic trifluoromethylation by substituting an aromatic amino group with a trifluoromethyl. This was a trifluoromethylation adaption of the classical Sandmeyer reaction.
To 180 (Scheme 58) dissolved in acetonitrile, was added dropwise a solution of tert-butyl nitrite and HCl to afford the diazonium salt 183. The change in Rf on TLC confirmed the presence of a new intermediate. To this, in situ generated AgCF$_3$, which was formed by the addition of silver fluoride into TMSCF$_3$, was added, however the reaction failed to afford 183a (Scheme 58).

**Trifluoromethylation using Ruppert-Prakash reagent**  

**Scheme 59. Synthesis of trifluoromethylcarbene 185**

![Scheme 59](image)

**Scheme 60. Attempted synthesis of 143**

Reagents and conditions: KF, CuI, TMSCF$_3$, NMP, 70 °C, 12 h
Then, trifluoromethylation was attempted using spray-dried potassium fluoride and copper iodide. TMSCF$_3$ Compound 184 with potassium fluoride leads to the formation of trifluoromethylcarbene 185 (Scheme 59) which then reacts with chloride 122 or iodide 142 but the reaction failed to afford 143 (Scheme 60).$^{139}$

Reason behind failure of the reaction: As seen in the earlier case with nitrile formation, chlorides are not good leaving groups, and so iodides were used this time. The reaction might have failed as the 6-C position is not electrophilic enough. Also super-dry potassium fluoride is required for this reaction, and spray drying was not possible in the lab conditions, and that might have been the reason behind reaction failure. Radical reactions (single electron transfer) could be tried next.

**Scheme 61. Attempted synthesis of 143**

![Scheme 61](image)

Reagents and conditions : CF$_3$SO$_3$-+S-(CF$_3$)(Ph)$_2$, Cu, DMF, 60 °C, 18 h

Another trifluoromethylation method used by Georgsson et al.$^{140}$ was attempted in which copper was added to a solution of diphenyl(trifluoromethyl)sulfonium trifluoromethanesulfonate and 142 in DMF under N$_2$ but was unsuccessful (Scheme 61)$^{245}$.

It was proposed (S)- (trifluoromethyl)diphenylsulfonium triflate is first reduced by copper
via single electron transfer which then decomposes rapidly to produce the CF$_3$ radical, which further generates CuCF$_3$.

**Scheme 62. Attempted synthesis of 143**

Reagents and conditions: NaSO$_2$CF$_3$, tBuOOH, H$_2$O, rt, 46 h

Stout et al.$^{141}$ attempted trifluoromethylation following the protocol of Baran and co-workers$^{142}$ (tBuOOH, aqueous NaSO$_2$CF$_3$, or ZnSO$_2$CHF$_2$) which smoothly converted the starting material to the trifluorinated analogues. However, when this method was attempted on the pyrimidine scaffold 137, it failed to afford 143 (Scheme 62).

Reason behind reaction failure: Both radical reactions failed to work. One reason might be that 6-C position is not electrophilic enough. As seen earlier in Suzuki or Negishi coupling case, reactions worked well when metals were used which could coordinate with pyrimidine nitrogen resulting in increased electrophilicity at 6-Cl.
Scheme 63. Synthesis of 143

Reagents and conditions: phenanthroline/CuCF$_3$ complex, DMF, 80 °C, 8 h, MW, 85 % yield

Trifluoromethylation was finally achieved when iodide 142 (Scheme 63) was treated with phenanthroline–CuCF$_3$ complex reagent 186, and DMF as solvent using microwave irradiation at 80 °C for 8 hours.$^{246}$

After attempting various aromatic substitution reactions, it was observed that-

1. Iodides or bromides should be used as they are better leaving groups compared to chlorides
2. Fluorides/chlorides are better for $S_{N}Ar$ reactions, as electrophilicity is more important there.
3. If iodides do not work, radical reactions could be attempted. To make position more electrophilic in pyrimidines, nitrogen could be coordinated with metals like zinc, copper which form a complex with nitrogen, and decrease electrophilicity of position next to it, facilitating substitution/coupling reactions.
Synthesis of 144-147

Appropriate 4,6-dichloropyrimidine-5-amines 190 and 191 (Scheme 64) were treated with 4-methoxyaniline and catalytic amount of conc. HCl to afford the corresponding 144 and 145 in 70-75 % yields. Compound 145 was then deprotonated with sodium hydride and treated with the appropriate alkyl halides to afford the corresponding N5-alkylated 146-147 in 35-65% yield.

Scheme 64. Synthesis of 144-147

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane b) NaH, MeI or EtI, DMF, 2 h, 0 °C-rt, 35-65 % yield.
**Synthesis of 148-150**

**Scheme 65. Synthesis of 149**

![Scheme 65 Synthesis of 149](image)

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 72 % yield  
b) NaH, MeI, DMF, 2 h, 0 °C-rt, 22 % yield

4,6-Dichloropyrimidine-2,5-diamine 187 (Scheme 65) was subjected to a nucleophilic aromatic substitution using 4-methoxyaniline and catalytic amount of conc. HCl in dioxane to afford 148 in 72 % yield. Reactions involving microwave irradiation rather than bench top worked with 15-25% better yields. Compound 148 was deprotonated with sodium hydride and treated with the appropriate alkyl hydride and treated with the appropriate alkyl halides to afford the corresponding N5-alkylated compounds 149-150 (Schemes 65 and 66). Reactions involving combinations of cesium carbonate or potassium carbonate and dimethylformamide with microwave irradiation did not show complete consumption of starting material. The use of excess base or alkyl halide also did not afford the alkylated product exclusively.
Scheme 66. Synthesis of 150

Reagents and conditions: a) N-methyl-4-methoxyaniline, cat. HCl (conc.), 2 h, 120 °C, MW, dioxane, 72 % yield  
   b) ethyl iodide, DMF, 2 h, 0° C-rt, 53 % yield

Two side products the 5-N,N-dimethylated 188 and the 2-N,5-N,N-trimethylated 189 were formed when 148 was deprotonated and treated with the methyl iodide which were easily isolated using column chromatography. The CHN elemental analysis confirmed the presence of 149 with C = 54.83%, H = 5.86%, N = 22.55%, Cl = 11.36 %. The $R_f$ of 149 was also 0.1-0.2 units lower than 188 and 189. In $^1$H NMR analysis, the 4-NH$_2$ peak of 148 can be seen at 4.7 ppm integrating for 2 protons, and the 2-NH$_2$ peak at 2.98 ppm (exact peak positions known as they were compared with 2-methyl and 4-methyl compounds). In 149, the 4-NH$_2$ gets methylated, and the peak shifts to 4.84 ppm integrating for only 1
proton. The 2-NH$_2$ peak shifts to 2.92 ppm still integrating for 2 protons. This confirmed that the methylation happened at 4-position.

**Synthesis of 151-155** : Starting materials 193 and 196 were synthesized for the synthesis of target compounds 151 and 153 respectively (Scheme 67). Compound 192 was first deprotonated with sodium hydride and treated with methyl iodide to afford the alkylated product 193 in 45% yield. However, an equal amount of dimethyl product 194 was formed as well. To obtain pure monomethylated product (193), 192 was treated with formaldehyde and sodium methoxide to yield the imine which was then reduced using sodium borohydride to afford pure 193 in 90% yield.

**Scheme 67. Synthesis of anilines 193 and 196.**
Reagents and conditions: a) NaH, MeI, DMF, 2 h, 0º C-rt b) i. (HCHO)$_n$, NaOMe, MeOH ii. NaBH$_4$, 85-90 % yield.

Compound 195 (Scheme 67) was treated with formaldehyde and sodium methoxide to yield imine which was then reduced using sodium borohydride to afford 196 in 85 % yield.

**Scheme 68. Synthesis of 151-155**

Reagents and conditions: appropriate anilines, cat. HCl (conc.), 2 h, 120 ºC, MW, dioxane, 20-85 % yield

Nucleophilic aromatic substitution reaction of 2-methyl-4,6-dichloro-5-aminopyrimidine 177 (Scheme 68) with appropriately substituted amines in dioxane afforded compounds 151-155 in 20-85% yields.\textsuperscript{229} Displacement reactions with bulky nucleophiles N-methyl-2,3-dihydrobenzofuran-5-amine and 6-methoxy-1,2,3,4-tetrahydroquinoline required
longer reaction times compared to the other amines for complete disappearance of starting material estimated from TLC. Displacement reactions with 4-methoxyaniline and 4-(methylthio)aniline afforded better yields than the methylated counterparts. Reactions involving microwave irradiation afforded 15-25% better yields than conventional bench-top conditions.

**Synthesis of 157-160**

Scheme 69. Synthesis of 157-160

![Chemical structures]

Reagents and conditions: appropriate anilines, cat. HCl (conc.), 5 h, 150 °C, MW, butanol, 32-65% yields.

**Table 14.** Chemical shift of 5-NH₂ protons using ¹H NMR (DMSO-d₆-400MHz)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift</th>
<th>Compound</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>3.99</td>
<td>155</td>
<td>5.18</td>
</tr>
<tr>
<td>151</td>
<td>4.32</td>
<td>156</td>
<td>3.04</td>
</tr>
<tr>
<td>152</td>
<td>4.50</td>
<td>159</td>
<td>3.5</td>
</tr>
<tr>
<td>153</td>
<td>3.98</td>
<td>160</td>
<td>3.72</td>
</tr>
</tbody>
</table>
A $^1$H NMR study was also done in DMSO-$d_6$ to compare the conformation of 122 with 154. The N-C4 bond (‘a’ bond, Scheme 68) connecting the phenyl ring and the pyrimidine ring in 164 is freely rotatable, while it is restricted in 122 due to the presence of 4N-Me group. According to the $^1$H NMR spectrum, the 5-NH$_2$ protons of 154 are less shielded (chemical shift at 5.11 ppm) than the 5-NH$_2$ of 4N-methylated 122 (chemical shift at 3.99 ppm). This suggested a nearby diamagnetic anisotropic cone in 122 which is missing in 154. Due to the bulk of the 4N-Me group, the conformation of 122 is restricted, and the phenyl ring of 122 positions itself on the top of the 5-NH$_2$ protons (conformation shown in Scheme 28) leading to the observed shielding effect. Due to the absence of 4N-Me group in 154, 4N-H places itself on the top of the 5-NH$_2$ protons, and so no shielding effect is observed as was found in the case of 122.\textsuperscript{83}

Some effects observed are-

1. The 5-NH$_2$ protons of all the final products are more shielded compared to the 5-NH$_2$ protons of the starting material 177 which come at 5.92 ppm. This suggested a nearby diamagnetic anisotropic cone due to the placement of aniline on top of 5-NH$_2$.

2. The 4-NMe compounds (122, 151-156, 159-160) are shielded compared to the 4N-H compounds 154 and 155. Due to the bulk of the 4N-Me group, the conformation of the final compounds (other than 154 and 155) is restricted, and so, the phenyl ring positions itself on the top of the 5-NH$_2$ protons leading to the observed shielding effect.\textsuperscript{83}

3. The disubstituted compounds 159 and 160 with 4,6-diMe are much more shielded
than their monosubstituted counterparts 122 and 151. This might have happened because both aniline rings might position themselves on the top of the 5-NH₂ leading to a greater diamagnetic anisotropic cone, and hence a stronger shielding effect.

**Synthesis of 162-167**

**Scheme 70. Synthesis of 198, 200 and 203a**

Reagents and conditions: a) i. (HCHO)$_n$, NaOMe, MeOH ii. NaBH₄, 75-85 % yields b) NaH, Mel, DMF, 2 h, 0º C-rt, 69%
Starting materials 198, 200 and 203a were synthesized for the synthesis of 163, 164 and 166 respectively (Scheme 70). Compounds 197, 199 and 201 were treated with formaldehyde and sodium methoxide to afford the imines which were then reduced using sodium borohydride to afford corresponding 198, 200 and 202 respectively in 75-85% yields. Compound 202 was then deprotonated with sodium hydride, and treated with the methyl iodide to afford the corresponding alkylated product 203a in 79% yield.

**Scheme 71. Synthesis of 206**

Reagents and conditions: a) 1. NaOMe, MeOH, rt, 30 min, 2. DMF, 100 °C, 6 h, b) NaOEt, EtOH, reflux, 4 h, Acetamidine HCl, c) H₂SO₄, EtOH, reflux, 2

To synthesize the pyrrolo[2,3-\(d\)]pyrimidine scaffold 206 (Scheme 71), the acidic proton of ethyl cyanoacetate 203b was deprotonated using sodium methoxide and alkylated to afford
which was then cyclized using acetamidine hydrochloride to yield 205. Under acidic conditions, 205 cyclized to afford 206.\(^{228, 247}\)

**Scheme 72. Synthesis of 162-167**

Reagents and conditions: appropriate anilines, Dioxane, HCl, 140 °C, 2 h, MW, 42-65% yield.

Direct cyclization of 6-amino-2-methylpyrimidin-4(3H)-one was also attempted with chloroacetaldehyde to afford 206, but was unsuccessful.\(^{228}\)
2-Methyl-pyrrolo[2,3-\(d\)]pyrimidine was subjected to nucleophilic aromatic substitution reaction using appropriate anilines and catalytic amount of conc. HCl in dioxane to afford 162-167 in 42-65% yields (Scheme 72).\(^{244}\)

A \(^1\)H NMR study was done in DMSO-\(d_6\) to determine the conformation of 162-167 and was compared with the starting material 206 as shown in Table 13.

**Table 15.** Chemical shift of aromatic 5-CH proton in \(^1\)H NMR (DMSO-\(d_6\)-400MHz)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift</th>
<th>Compound</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td>6.52</td>
<td>165</td>
<td>5.9</td>
</tr>
<tr>
<td>162</td>
<td>4.6</td>
<td>166</td>
<td>4.45</td>
</tr>
<tr>
<td>163</td>
<td>4.75</td>
<td>167</td>
<td>4.65</td>
</tr>
<tr>
<td>164</td>
<td>6.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The aromatic 5-CH proton of all the final products is more shielded compared to the same proton in the starting material 206 suggesting a nearby diamagnetic anisotropic cone. So, that’s why, we believe that the phenyl ring positions itself on the top of the 5-CH proton leading to the observed shielding effect.\(^{83}\)

**Synthesis of 173-174**

Commercially available esters 207a-207b (Scheme 73) were alkylated with 6-bromo-1-hexanol to afford alkylated intermediates 208a-208b. The intermediates were then oxidized using Dess-Martin Oxidation to the respective aldehydes 209a-209b. \(\alpha\)-
Bromination of the aldehydes with bromine-dioxane mixture at 0 °C afforded the corresponding α-bromo aldehydes 210a-210b. The

**Scheme 73. Synthesis of 173-174**

Reagents and conditions: a) K$_2$CO$_3$, DMF, rt, 10 h b) Dess-martin reagent, DCM, rt, 5 h c) Br$_2$, dioxane, DCM, rt, 2 h d) H$_2$O/MeOH 1:1, CH$_3$COONa, 50° C, 5 h e) 1 N NaOH f) NMM, CDMT, DMF, rt, 5 h g) 1 N NaOH

Commercially available esters 207a-207b (Scheme 73) were alkylated with 6-bromo-1-hexanol to afford alkylated intermediates 208a-208b. The intermediates were then oxidized using Dess-Martin Oxidation to the respective aldehydes 209a-209b. α-
Bromination of the aldehydes with bromine-dioxane mixture at 0 °C afforded the corresponding α-bromo aldehydes 210a-210b. The 5-substituted pyrrolo[2,3-$d$]pyrimidines 211a-211b were then synthesized by condensation of 210a-210b with 2,4-diamino-6-hydroxypyrimidine at 45 °C in the presence of sodium acetate. Subsequent hydrolysis with 3 N NaOH afforded key intermediates 2-amino-4-oxo-pyrrolo[2,3-$d$]pyrimidin-5-yl-alkoxybenzoic acids 211a-211b, followed by coupling with (L)-diethyl glutamate esters using N-methylmorpholine and 6-chloro-2,4-dimethoxytriazine as the activating agents, to afford diesters 212a-212b. Final saponification of the diesters with 1 N NaOH yielded the target 5-substituted pyrrolo[2,3-$d$]pyrimidines 173-174.
Synthesis of 175-176

Scheme 74. Synthesis of 215

Reagents and conditions: a) K$_2$CO$_3$, MeCN, rt, 10 h
b) Dess-martin reagent, DCM, rt, 5 h
c) 1. oxalyl chloride, DMSO, THF  2. TEA
d) 1. NaBr, TEMPO, DCM/H$_2$O (4:1), 0 °C  2. 6% NaOCl, NaHCO$_3$, 0 °C, 2 h.
Scheme 75. Synthesis of 218

Reagents and conditions: a) EtOH, H$_2$SO$_4$, reflux, 6 h b) Cs$_2$CO$_3$, DMF, rt, 10 h c) Dess-martin reagent, DCM, rt, 5 h d) 1. NaBr, TEMPO, DCM/H$_2$O (4:1), 0 °C 2. 6% NaOCl, NaHCO$_3$, 0 °C, 2 h e) PCC, DCM, 30 min, rt

For the synthesis of the target compound 175, compound 207a (Scheme 74) was alkylated with 4-bromo-1-butanol to afford alkylated intermediates 214. The intermediate were then oxidized using Dess-Martin Oxidation but failed to afford 215. Similarly, for the synthesis of target compound 176, compound 207b (Scheme 75) was alkylated with 4-bromo-1-
butanol to afford alkylated intermediates 217. Dess-Martin oxidation failed to afford 218 and so different oxidation methods were attempted for the oxidation of both 214 and 217 as summarized in Table 14

Table 16. Different oxidation methods used and their results.

<table>
<thead>
<tr>
<th>Type</th>
<th>Reagents</th>
<th>Temperature</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP</td>
<td>DMP</td>
<td>rt</td>
<td>Multiple spots</td>
</tr>
<tr>
<td>Swern</td>
<td>DMSO, Oxalyl chloride, TEA</td>
<td>- 78 ºC</td>
<td>Multiple spots</td>
</tr>
<tr>
<td>TEMPO</td>
<td>TEMPO, 6% NaOCl, NaHCO₃</td>
<td>0 ºC</td>
<td>215</td>
</tr>
<tr>
<td>PCC</td>
<td>PCC in DCM</td>
<td>rt</td>
<td>218</td>
</tr>
</tbody>
</table>

Swern oxidation afforded multiple products which were difficult to isolate using column chromatography. TEMPO oxidation worked successfully to afford 215 (Scheme 74). Compound 217 under TEMPO oxidation conditions cleaved to yield alcohol 216 (Scheme 75). PCC oxidation was attempted which worked successfully to afford 218 (Scheme 75).

With these reactions, it was observed that for heteroatoms, various kind of oxidation reactions should be attempted. Different reaction conditions ranging from - 78 ºC to rt should be tried. Different reactions could also range from partially acidic to basic conditions. TEMPO reaction is run under partial acidic conditions, DMP under neutral, and Swern under basic conditions as TEA is used. Sulfur as a heteroatom could have formed a
complex with DMP, and this might be the reason behind failure of the reaction. With PCC, that complex did not form, and reaction worked successfully.

**Synthesis of 175**

α-Bromination of the aldehyde 215 (Scheme 76) with bromine-dioxane mixture at 0 °C afforded the α-bromo aldehydes 216 (Scheme 76). The 5-substituted pyrrolo[2,3-d]pyrimidines 217 was then synthesized by condensation of 216 with 2,4-diamino-6-hydroxypyrimidine at 45 °C in the presence of sodium acetate. Subsequent hydrolysis with 3 N NaOH afforded key intermediate 2-amino-4-oxo-pyrrolo[2,3-d]pyrimidin-5-yl-alkoxybenzoic acid 218, followed by coupling with (L)-diethyl glutamate esters using N-methylmorpholine and 6-chloro-2,4-dimethoxytriazine as the activating agents, to afford diesters 219. Final saponification of the diesters with 1 N NaOH yielded the target 5-substituted pyrrolo[2,3-d]pyrimidines 175.
Scheme 76. Synthesis of 175

Reagents and conditions: c) Br$_2$, dioxane, DCM, rt, 2 h d) H$_2$O/MeOH 1:1, CH$_3$COONa, 50° C, 5 h e) 1 N NaOH f) NMM, CDMT, DMF, rt, 5 h g) 1 N NaOH
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. 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 with TopSpin 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. Thin-layer chromatography (TLC) was performed on Whatman® PE SIL G/UV254 flexible silica gel plates 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. Column chromatography was performed on the silica gel (70 to 230 meshes, Fisher Scientific) column. Flash chromatography was carried out on the CombiFlash® Rf systems, model COMBIFLASH RF. Pre-packed 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. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.
compositions are within ± 0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented despite 24 to 48 hours of drying in vacuo and were confirmed where possible by their presence in the $^1$H NMR spectra. High Performance Liquid Chromatography (HPLC) was performed on Waters® 600E Multisolvent Delivery System, components: Waters® 600 Controller (model code: 600), 600E Pump (model code: 60F), Waters® 2487 Dual λ Absorbance Detector. Mobile phase was an aqueous blend of water with a miscible, polar organic solvent acetonitrile.

**6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidin-4,5-diamine (122)**

2-Methyl-4,6-dichloropyrimidine (2g, 11.23 mmol, 1 equiv) and the N-methyl-4-methoxyaniline (13.48 mmol, 1.2 equiv) were dissolved in dioxane (15 mL). To this solution, was added 37% HCl (cat.). The mixture was heated at 120 ºC in microwave and stirred for 1.5 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of aq. NH$_4$OH. The butanol is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brown solid in 75% yield. TLC $R_f$ 0.5 (EtOAc: Hex; 1:1), $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 2.55 (s, 3H, 2CH$_3$), 3.44 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4’OCH$_3$), 2.55 (s, 2H, NH$_2$), 6.90-6.92 (d, 2H, J = 8.8 Hz), 7.06-7.08 (d, 2H, J = 8.8 Hz), Anal. calcd. for C$_{13}$H$_{15}$ClN$_4$O: C = 56.02%, H = 5.42%, N = 20.10%, Cl = 12.72%, Found C = 56.29%, H = 5.51%, N = 20.00%, Cl = 12.55%, M.P.= 109-110º C.
6-chloro-N4-(4-methoxyphenyl)-N4,N5-2-trimethylpyrimidine-4,5-diamine (123)

Sodium hydride (29 mg, 1.1 equiv, 1.18 mmol) was added to a 3-neck flask. DMF (10 mL) was added under inert conditions. The N-methyl-4-methoxyaniline (300 mg, 1 equiv, 1.08 mmol) compound is then added at 0 °C. After 20 minutes when the nucleophile is generated, methyl iodide (260 mg, 1.5 equiv, 1.84 mmol) is added dropwise and reaction was run at room temperature for 2 hours. DMF is then stripped off and the reaction mixture was neutralized to pH 8. The compound was then extracted in the organic layer using ether and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 35 % yield. TLC $R_f$ 0.62 (EtOAc: Hex; 1:1), $^1$H NMR (400 MHz, DMSO-d6): $\delta$ 2.26 (s, 3H, 5-NHCH$_3$), 2.39 (s, 3H, 2CH$_3$), 3.31 (s, 3H, 4NCH$_3$), 3.74 (s, 3H, 4'OCH$_3$), 6.87-6.89 (d, 2H, J = 8.8 Hz), 6.98-7.00 (d, 2H, J = 8.8 Hz), Anal. calculated for C$_{14}$H$_{17}$ClN$_4$O : C = 58.03%, H = 6.09%, N = 18.69%, Cl = 11.83%, Found C = 58.06%, H = 5.97%, N = 18.76%, Cl = 11.85%, M.P. = 67-68° C.

6-chloro-N4-(4-methoxyphenyl)-N4,N5,N5,2-tetramethylpyrimidine-4,5-diamine (124)

Sodium hydride (29 mg, 1.1 equiv, 1.18 mmol) was added to a 3-neck flask. DMF (10 mL) was added under inert conditions. The N-methyl-4-methoxyaniline (300 mg, 1 equiv, 1.08 mmol) compound is then added at 0 °C. After 20 minutes when the nucleophile is generated, the alkyl halide (252 mg, 1.5 equiv, 1.61 mmol) is added dropwise and reaction was run at room temperature for 2 hours. DMF is then stripped off and the reaction mixture
was neutralized to pH 8. The compound was then extracted in the organic layer using ether and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brown solid in 50 % yield. TLC $R_f$ 0.65 (EtOAc: Hex; 1:1), $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 2.14 (s, 6H, 5-N-diCH$_3$), 2.54 (s, 3H, 2CH$_3$), 3.37 (s, 3H, 4NCH$_3$), 3.83 (s, 3H, 4'OCH$_3$), 6.87-6.89 (d, 2H, J = 8.8 Hz), 7.08-7.10 (d, 2H, J = 8.8 Hz). Anal. calculated for C$_{15}$H$_{19}$ClN$_4$O: C, 59.27; H, 6.46; N, 17.86; Cl, 11.30, Found C = 59.16%, H = 6.37%, N = 17.71%, Cl = 11.23%, M.P. = 71-73º C.

**6-chloro-N5-ethyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine**

(125)

Compound 122 (100 mg, 0.25 mmol) was taken in a 3-neck round bottom flask covered with a septum, an adapter and a stopper. NaH (9.47 mg, 1.1 equiv) was added quickly and was diluted with 3 mL of DMF. The reaction is kept under argon for the entire time. After 10 min, ethyl iodide (84 mg, 1.5 equiv, 0.54 mmol) was added and remaining DMF (2 mL) was added quickly after that. The reaction was allowed to run under argon for 4 hrs at room temperature. DMF was stripped off using high pressure. The product was then extracted using ethyl acetate and dried under sodium sulfate for half an hour. Ethyl acetate was then evaporated using rotary evaporator at 36 ºC. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 68 % yield. TLC $R_f$ 0.72 (EtOAc: Hex; 1:1), $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 0.64 (t, 3H, CH$_3$), 2.40 (s, 3H, 2-CH$_3$), 2.62-2.64 (q, 2H, 5-N-CH$_2$), 3.74 (s, 3H, NCH$_3$), 3.32 (s, 3H, OCH$_3$), 6.88-6.90 (d, 2H, J = 8.8
Hz), 7.02-7.04 (d, 2H, J = 8.8 Hz), Anal. calculated for C_{16}H_{21}ClN_{4}O: C, 58.73; H, 6.24; N, 18.26; Cl, 11.55; Found C = 58.70%, H = 6.22%, N = 18.38%, Cl = 11.46%, M.P. = 84-85°C.

6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethyl-N5-propylpyrimidine-4,5-diamine (126·HCl)

Sodium hydride (29 mg, 1.1 equiv, 1.18 mmol) was added to a 3-neck flask. DMF (10 mL) was added under inert conditions. The N-methyl-4-methoxyaniline (300 mg, 1 equiv, 1.08 mmol) compound is then added at 0 °C. After 20 minutes when the nucleophile is generated, propyl iodide (252 mg, 1.5 equiv., 1.61 mmol.) is added dropwise and reaction was run at room temperature for 2 hours. DMF is then stripped off and the reaction mixture was neutralized to pH 8. The compound was then extracted in organic layer using ether and dried under magnesium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brownish semi solid in 78 % yield. The product was converted into a HCl salt using HCl gas in ether solution. TLC $R_f$ 0.88 (EtOAc: Hex; 1:1), $^1$H NMR (DMSO-d$_6$, 400MHz): δ 0.65(t, 3H,CH$_3$, J = 7.6 Hz), 0.98-1.02 (m, 2H, CH$_2$), 2.40 (s, 3H, 2-CH$_3$), 2.52-2.55 (m, 2H, CH$_2$, J = 7.6 Hz), 3.33 (s, 3H, 4‘OCH$_3$), 3.45 (s, 1H, 5-NH), 3.74 (s, 3H, 4NCH$_3$), 6.87-6.89 (d,2H, J = 8.8 Hz), 6.99-7.01 (d,2H, J = 8.8 Hz), Analysis calculated for C$_{16}$H$_{21}$ClN$_4$O.0.4H$_2$O.1.0HCl, C = 52.72%, H = 6.31, N = 15.37%, Cl = 19.45%, Found C = 53.04%, H = 5.92, N = 15.34%, Cl = 19.13%, M.P. for 126·HCl = 107°C.
**N5-buty1-6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (127·HCl)**

Sodium hydride (29 mg, 1.1 equiv, 1.18 mmol) was added to a 3-neck flask. DMF (10 mL) was added under inert conditions. The aniline (300 mg, 1 equiv, 1.08 mmol) compound is then added at 0 °C. After 20 minutes when the nucleophile is generated, butyl iodide (252 mg, 1.5 equiv, 1.61 mmol) is added dropwise and reaction was run at room temperature for 2 hours. DMF is then stripped off and the reaction mixture was neutralized to pH 8. The compound was then extracted in organic layer using ether and dried under magnesium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brownish semisolid in 62 % yield. The product was converted into a HCl salt using HCl gas in ether solution. TLC Rf 0.90 (EtOAc: Hex; 1:1) 1H NMR (CDCl3, 400MHz): δ 0.80-0.84 (t, 3H, CH3, J = 7.6 Hz), 1.05-1.17 (m, 4H, CH2), 2.56(s, 3H, CH2), 2.65-2.67 (m, 2H, CH2), 2.84 (s, 1H, NH), 3.43 (s, 3H, 4NCH3), 3.83 (s, 3H, 4’OCH3), 6.87-6.89 (d,2H, J = 8.8 Hz), 6.99-7.01 (d, 2H, J = 8.8 Hz). Analysis calculated for (C17H23ClN4O.1.0HCl) C = 55.14%, H = 6.26, N = 15.13%, Cl = 19.15%. Found C = 54.86%, H = 6.52, N = 15.08%, Cl = 18.92%, M.P. for 127·HCl = 119-123 °C.

**6-chloro-N5-isobuty1-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (128)**

Sodium hydride (29 mg, 1.1 equiv, 1.18 mmol) was added to a 3-neck flask. DMF (10 mL) was added under inert conditions. The aniline (300 mg, 1 equiv, 1.08 mmol) compound is
then added at 0 °C. After 20 minutes when the nucleophile is generated, isobutyl iodide (252 mg, 1.5 equiv, 1.61 mmol) is added dropwise and reaction was run at room temperature for 2 hours. DMF is then stripped off and the reaction mixture was neutralized to pH 8. The compound was then extracted in organic layer using ether and dried under magnesium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown semi-solid in 65 % yield. TLC \( R_f \) 90 (EtOAc: Hex; 1:1) \(^1\)H NMR (CDCl\(_3\), 400MHz): \( \delta \) 0.72 (s, 3H, CH\(_3\)), 0.74 (s, 3H, CH\(_3\)), 1.25-1.33 (m, 1H, CH), 2.49-2.52 (t, 2H, CH\(_2\)), 2.55 (s, 3H, CH\(_3\)), 2.96 (m, 1H, NH), 3.44 (s, 3H, 4NCH\(_3\)), 3.82 (s, 3H, 4’OCH\(_3\)), 6.87-6.89 (d, 2H, \( J = 8.8 \) Hz), 6.99-7.01 (d, 2H, \( J = 8.8 \) Hz). HPLC –98.47% pure, LRMS calculated for C\(_{17}\)H\(_{23}\)ClN\(_4\)O = 334.85, found 334.85.

6-benzyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (130)

0.5 M solution of benzyl zinc chloride (5.7 mL, 4 equiv, 2.87 mmol) and tetrakis(triphenylphosphine)palladium (0.05 equiv, 36 µmol) was added to a sloution of iodopyrimidine (1 equiv, 540 µmol) in dry THF at 60 °C for 12 h under inert conditions. The reaction was stopped and THF was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brown semisolid in 35 % yield. TLC \( R_f \) 0.20 (EtOAc: Hex; 1:1) \(^1\)H NMR (DMSO-\( d_6\), 400MHz): \( \delta \) 2.57 (s, 3H, 2CH\(_3\)), 3.49 (s, 3H, 4NCH\(_3\)), 3.79 (s, 3H, 4’OCH\(_3\)), 4.08 (s, 2H, CH\(_2\)), 4.23 (s, 2H, NH\(_2\)), 6.99-7.01 (d, 2H, aromatic), 7.22-
7.32 (m, 7H, aromatic). HPLC -97.23% pure, HRMS calculated for C₂₀H₂₅N₄O [M + H]^+ 335.1874, found 335.1862.

**N₄-(4-methoxyphenyl)-N₄,2-dimethyl-6-phenethylpyrimidine-4,5-diamine (131)**

The aniline 152 (1g, 2.9 mmol, 1 equiv) was hydrogenated using 5% Pd/C (200 mg, 20% w/w) in 15 mL methanol for 7 h under 50 psi. Palladium was then filtered using Celite® and washed several times with methanol. Methanol was then striped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 131 in 35 % yield as a brownish semisolid. TLC Rₜ 0.35 (EtOAc: Hex, 1:1); ^1H NMR (400 MHz, DMSO-d₆): δ 2.41 (s, 3H), 2.80-2.84 (dd, J = 6.0Hz, J = 9.6Hz, 2H), 2.92-2.96 (dd, J = 6.1Hz, J = 10.2Hz, 2H), 3.25 (s, 3H), 3.73 (s, 3H), 3.85 (s, 2H), 6.88 (m, 4H), 7.18 (m, 1H), 7.26 (s, 2H), 7.27 (s, 2H), HPLC -96.9% pure, HRMS calculated for C₂₁H₂₅N₄O [M + H]^+ 349.2031, found 349.2030.

**N₄-(4-methoxyphenyl)-N₄,2-dimethyl-6-(phenylethynyl)pyrimidine-4,5-diamine (132)**

Copper iodide (0.16 equiv, 3.38 mmol) and bis(triphenylphosphine)palladium chloride (0.05 equiv, 67.5 mmol) were added to a solution of iodopyridine (1 equiv, 1.35 mmol) in 10 mL trimethylamine under nitrogen gas and the resulted compound was stirred for 30 min. Then phenylacetylene (2.5 equiv, 3.38 mmol) was added dropwise to the solution, and was stirred for 30 minutes. Silica gel was added and the solvent evaporated to obtain a
plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 132 in 65% yield as a dark brown solid. TLC Rf 0.50 (EtOAc: Hex, 1:1); \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) 2.41 (s, 3H), 3.34 (s, 3H), 3.76 (s, 3H), 4.19 (s, 2H), 6.95-6.97 (d, \(J = 8.9\) Hz, 2H), 7.07-7.09 (d, \(J = 8.9\) Hz, 2H), 7.42 (d, \(J = 1.3\) Hz, 1H), 7.44 (d, \(J = 2.3\) Hz, 1H), 7.63-7.66 (m, 2H) Anal. calcd. for C\(_{21}\)H\(_{20}\)N\(_4\)O: C, 73.23; H, 5.85; N, 16.27; Found C = 73.13%, H = 5.90, N = 16.11%.

\(\text{N-(4-chloro-6-((4-methoxyphenyl)(methyl)amino)-2-methylpyrimidin-5-yl)acetamide (133)}\)

Compound 122 (50 mg, 0.18 mmol) was dissolved in 6 mL acetic anhydride and the reaction was run at 120 °C for 5 h. The solvent was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a white solid in 75% yield. TLC Rf 0.25 (EtOAc: Hex; 1:1), \(^1\)H NMR (DMSO-d\(_6\), 400 MHz): \(\delta\) 1.6 (s, 3H, NAc.), 2.61 (s, 3H, 2CH\(_3\)), 3.42 (s, 3H, 4NCH\(_3\)), 3.82 (s, 3H, 4’OCH\(_3\)), 5.70 (s, 1H, NH), 6.94-6.96 (d, 2H, \(J = 8.8\) Hz), 7.22-7.25 (d, 2H, \(J = 8.8\) Hz) Analysis calculated for C\(_{15}\)H\(_{17}\)ClN\(_4\)O\(_2\) : C = 56.16%, H = 5.34, N = 17.47%, Cl = 11.05%; Found C = 56.33%, H = 5.52, N = 17.37%, Cl = 10.88%, M.P. = 158-159 °C.
\(N\)-acetyl-\(N\)-(4-chloro-6-\(\text{(4-methoxyphenyl)(methyl)amino}\))-2-methylpyrimidin-5-yl)acetamide (134)

Compound 122 (50 mg, 0.18 mmol) was dissolved in acetic anhydride (6 mL) and the reaction was run at 150 ºC for 5 h. The solvent was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a white solid in 45 % yield. TLC \(R_f\) 0.75 (EtOAc: Hex; 1:1); \(^1\)H NMR (CDCl\(_3\), 400MHz): \(\delta\) 1.99 (s, 6H, NPiv.), 2.61 (s, 3H, 2CH\(_3\)), 3.42 (s, 3H, 4NCH\(_3\)), 3.82 (s, 3H, 4’OCH\(_3\)), 6.94-6.96 (d, 2H, \(J = 8.8\) Hz), 7.22-7.25 (d, 2H, \(J = 8.8\) Hz). Analysis calculated for C\(_{17}\)H\(_{19}\)ClN\(_4\)O\(_3\): C, 56.28; H, 5.28; N, 15.44; Cl, 9.77; Found C = 56.37%, H = 5.33%, N = 15.41%, Cl = 9.59%, M.P. = 179-181 ºC.

\(N\)-(4-chloro-6-\(\text{(4-methoxyphenyl)(methyl)amino}\))-2-methylpyrimidin-5-yl)pivalamide (135)

Compound 122 (50 mg, 0.18 mmol) was dissolved in pivalic anhydride (6 mL) and the reaction was run at 120 ºC for 2 h. The solvent was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a grey solid in 78 % yield. TLC \(R_f\) 0.28 (EtOAc: Hex; 1:1); \(^1\)H NMR (DMSO-\(d_6\), 400MHz): \(\delta\) 1.39 (s, 9H, CH\(_3\)), 2.55 (s, 3H, 2CH\(_3\)), 3.78 (s, 3H, 4NCH\(_3\)), 3.87 (s, 3H, 4’OCH\(_3\)), 6.94-6.96 (d, 2H, \(J = 8.8\) Hz), 7.22-7.25 (d, 2H, \(J = 8.8\) Hz). Analysis calculated for C\(_{18}\)H\(_{23}\)ClN\(_4\)O\(_2\): C, 59.58; H, 6.39; N, 15.44; Cl, 9.77; Found C = 59.57%, H = 6.45, N = 15.25%, Cl = 9.65%, M.P. = 193-194º C.
6-chloro-N-(4-methoxyphenyl)-N,2-dimethyl-5-nitropyrimidin-4-amine (136)

2-methyl-4,6-dichloro-5-nitropyrimidine (1 g, 4.81 mmol), 4-methoxyaniline (792 mg, 5.77 mmol, 1.2 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 120 °C for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a yellowish solid in 82 % yield. TLC R$_f$ 0.70 (EtOAc: Hex; 1:1) $^1$H NMR (DMSO-$d_6$, 400MHz): δ 2.62 (s, 3H, CH$_3$), 3.53 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4’OCH$_3$), 6.86-6.88 (d, 2H, J = 8.8 Hz), 7.07-7.09 (d, 2H, J = 8.8 Hz). Analysis calculated for C$_{13}$H$_{13}$ClN$_4$O$_3$: C, 52.95; H, 5.11; N, 16.84; Cl, 10.65; Found C = 53.04%, H = 4.74, N = 16.87%, Cl = 10.46%, M.P. = 91-93° C.

N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (137)

The aniline 122 (50 mg, 0.18 mmol) was hydrogenated using 5% Pd/C (10 mg, 20% w/w) in methanol (10 mL) for 7 h under 50 psi. Palladium was then filtered using Celite® and washed several times with methanol. Methanol was then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 65 % yield. TLC R$_f$ 0.40 (EtOAc: Hex; 1:1) $^1$H NMR (400 MHz, CDCl$_3$): δ 2.70 (s, 3H, 2CH$_3$), 3.54 (s, 3H, 4NCH$_3$), 3.86 (s, 3H, 4’OCH$_3$), 4.22 (s, 2H, NH$_2$), 6.92-6.94 (d, 2H, J = 8.8 Hz), 7.15-7.17
(d, 2H, J = 8.8 Hz), 8.15 (s, 1H), (C_{13}H_{16}N_{4}O), HPLC –99.2% pure, HRMS calculated for C_{13}H_{17}N_{4}O [M + H]^+ 245.1404, found 245.1404, M.P. = 183-185º C.

**N^4-(4-methoxyphenyl)-N^4,2,6-trimethylpyrimidine-4,5-diamine (138)**

To a solution of 153 in 10 mL THF was added trimethylaluminium (1M solution in THF, 2 equiv, 718 µmol) and tetrakis(triphenylphosphine)palladium (0.1 equiv) and reaction was run under reflux and inert conditions. THF was then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 65 % yield. TLC R_f 0.6 (EtOAc: Hex; 1:1), ^1H NMR (400 MHz, DMSO-d6): δ 2.18 (s, 3H, 6CH₃), 2.37 (s, 3H, 2CH₃), 3.24 (s, 3H, 4NCH₃), 3.75 (s, 3H, 4’OCH₃), 3.81(s, 2H, NH₂), 6.89 (m, 4H), HPLC –97.8% pure, C_{14}H_{16}N_{4}O [M + H]^+ 259.1561 found 259.1548, M.P. 108-110 ºC.

**6-ethyl-N^4-(4-methoxyphenyl)-N^4,2-dimethylpyrimidine-4,5-diamine (139·HCl)**

To a solution of 153 in THF was added triethylaluminium (1M solution in THF, 2 equiv, 718 µmol) and tetrakis(triphenylphosphine)palladium (0.1 equiv) and reaction was run under reflux and inert conditions. THF was then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown semisolid in 58 % yield. TLC R_f 0.68 (EtOAc: Hex; 1:1), ^1H NMR (400 MHz, DMSO-d6): δ 1.25 (s, 3H), 2.58 (s, 2H), 2.59 (s, 3H, 2CH₃), 3.00 (s, 2H, NH₂), 3.43 (s, 3H, 4NCH₃), 3.82 (s, 3H, 4’OCH₃), 6.88-6.90 (d, 2H, J = 8.8 Hz), 6.99-7.01 (d, 2H, J = 8.8 Hz), C_{15}H_{20}N_{4}O, HPLC –99.5% pure, HRMS
calculated for C\textsubscript{15}H\textsubscript{21}N\textsubscript{4}O [M + H]\textsuperscript{+} 273.1717, found 273.1729, M.P. for 139·HCl = 200-203 ºC.

\textit{N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5,6-triamine (140)}

6-chloro-2-methylpyrimidine-4,5-diamine (50 mg, 1 equiv, 315 µmol), 4-methoxy aniline (65 mg, 1.5 equiv, 473 µmol) and catalytic amount of conc. HCl (1-2 drops) were added to a microwave vial in 15 mL dioxane and heated at 120 ºC for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl\textsubscript{3} followed by 3% MeOH in CHCl\textsubscript{3} and then 5% MeOH in CHCl\textsubscript{3}. Fractions containing the product were pooled and evaporated to afford a white solid in 42 % yield. TLC \(R_f\) 0.75 (CH\textsubscript{3}OH: CHCl\textsubscript{3}; 1:5), \(^1\)H NMR (DMSO-\textit{d}_6, 400MHz): \(\delta\) 2.22 (s, 3H, CH\textsubscript{3}), 3.15 (s, 3H, 4NCH\textsubscript{3}), 3.70 (s, 3H, 4′OCH\textsubscript{3}), 3.70-3.75 (s, 2H, 5NH\textsubscript{2}), 6.31 (s, 2H, 6NH\textsubscript{2}), 6.71-6.73 (d, C′2, 6 aromatic), 6.81-6.83(d, C′3, 5), Analysis calculated for C\textsubscript{13}H\textsubscript{17}N\textsubscript{5}O. 0.23 H\textsubscript{2}O: C, 60.21; H, 6.61; N, 27.01, Found C = 59.17%, H = 6.41, N = 26.76%, M.P. = 179-181 ºC.

\textit{5-amino-6-((4-methoxyphenyl)(methyl)amino)-2-ethylypyrimidine-4-carbonitrile (141)}

Iodopyridine 142 (0.45 g, 1.22 mmol) was dissolved in 10 mL DMF and reaction is heated to 120 ºC with stirring and copper cyanide (3 equiv, 3.65 mmol) was added. There was complete conversion in 10 min. The reaction mixture was cooled to room temp. and filtered using Buchner funnel. Silica gel was added and the solvent evaporated to obtain a plug.
Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 141 in 22% yield as a yellow solid: TLC R_f 0.66 (EtOAc: Hex, 1:1); ^1H NMR (400 MHz, DMSO-d6): δ 2.40 (s, 3H), 3.33 (s, 3H), 3.77 (s, 3H), 4.67 (s, 2H), 6.96-6.98 (d, J = 8.9 Hz, 2H), 7.13-7.15 (d, J = 8.9 Hz, 2H) Anal. calcd. for C_{14}H_{15}N_5O. 0.1 H_2O: C, 62.02; H, 5.65; N, 25.83, Found C = 61.99%, H = 5.52%, N = 25.58%, M.P. = 126.0-129.0 ºC.

6-iodo-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (142)

Pyrimidine 122 (1g, 3.59 mmol) was added in portions in stirring aqueous HI (57 wt % aq., 25 mL) at 0 ºC and then stirred overnight. The reaction mixture was then neutralized by adding 15 mL of ammonium hydroxide and then extracted using ethyl acetate and dried under magnesium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 0.92 g (65%) as a greenish solid: TLC R_f 0.65 (EtOAc: Hex, 1:1); ^1H NMR (CDCl_3) δ ^1H NMR (400 MHz, CDCl_3) δ 2.56 (s, 3H), 3.43 (s, 3H), 3.83 (s, 3H), 3.90 (s, 2H), 6.88-6.89 (d, J = 8.8 Hz, 2H), 7.02-7.03 (d, J = 8.8 Hz, 2H) Anal. calcd. for C_{13}H_{15}IN_4O: C, 42.18; H, 4.08; N, 15.13; I, 34.28, Found C = 42.48%, H = 4.24%, N = 15.15%, I = 34.15%, M.P. = 88.8-91.0 ºC.
\textit{N4-(4-methoxyphenyl)-N4,2-dimethyl-6-(trifluoromethyl)pyrimidine-4,5-diamine (143)}

Trifluoromethylation was done using phenanthroline–CuCF$_3$ complex reagent (1.3 equiv, 122 mg, 0.389 mmol) and 10 mL DMF as solvent in microwave at 80 °C in 8 hours. DMF is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 143 in 85% yield as a dark brown solid: TLC R$_f$ 0.82 (EtOAc: Hex, 1:1); $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 2.43 (s, 3H), 3.30 (s, 3H), 3.74 (s, 3H), 4.35 (s, 2H), 6.93-6.96 (dd, J = 3.6Hz, J = 8.8Hz, 2H), 7.06-7.08 (dd, J = 3.5Hz, J = 8.9Hz, 2H) HPLC – 98.8% pure, HRMS calculated for C$_{14}$H$_{15}$F$_3$N$_4$O [M + H]$^+$ 313.1278, found 313.1261, M.P. = 125-126 °C.

\textit{6-chloro-N4-(4-methoxyphenyl)-N4-methylpyrimidine-4,5-diamine (144)}

4,6-dichloropyrimidin-5-amine (80 mg, 1equiv, 485 µmol), 4-methoxyaniline (101 mg, 0.73 mmol, 1.5 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 120 °C for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a grey solid in 68 % yield. TLC R$_f$ 0.39 (EtOAc: Hex; 1:1), $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 3.46 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4'$\text{OCH}_3$), 3.5-3.8 (s, 2H, 5NH$_2$), 6.90-6.91 (d, C’2, 6 aromatic), 7.06-7.07 (d, C’3, 5), 8.2 (s, 1H, 2-H), Anal.
calcd. for C₁₂H₁₃ClN₄O: C, 54.45; H, 4.95; N, 21.17; Cl, 13.39; Found C = 54.72%, H = 5.00%, N = 20.93%, Cl = 13.12%, M.P. = 126.0-129.0 °C.

6-chloro-2-ethyl-N₄-(4-methoxyphenyl)-N₄-methylpyrimidine-4,5-diamine (145·HCl)

2-ethyl-4,6-dichloropyrimidine (100 mg, 0.52 mmol, 1 equiv.) and the N-methyl-4-methoxyaniline (0.62 mmol, 1.2 equiv.) were dissolved in butanol (15 mL). To this solution, was added 37% HCl (cat.). The mixture was heated at 120 °C in microwave with stirring for 1.5 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of aq. NH₄OH. The butanol is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a grey solid in 68 % yield. TLC Rf 0.75 (EtOAc: Hex; 1:1), ¹H NMR (CDCl₃, 400MHz):  δ 1.35 (s, 3H, 2CH₃), 2.84 (s, 2H, 2CH₂), 3.48 (s, 3H, 4NCH₃), 3.84 (s, 3H, 4’OCH₃), 6.92-6.94 (d, 2H, J = 8.8 Hz), 7.07-7.08 (d, 2H, J = 8.8 Hz), Anal. calcd. for C₁₄H₁₇ClN₄O.0.95H₂O. 0.95HCl: C, 48.80; H, 5.80; N, 16.26; Cl, 20.07; Found C = 48.92%, H = 5.82%, N = 15.94%, Cl = 20.04 %, M.P. = 135-136.0 °C.

6-chloro-2-ethyl-N₄-(4-methoxyphenyl)-N₄,N₅-dimethylpyrimidine-4,5-diamine (146)

Compound 145 (100 mg, 1 equiv, 0.36 mmol) was taken in a 3 necked round bottom flask covered with septums, and adapter. NaH (1.1 equiv, 0.39 mmol) was added and was diluted with 3 mL of DMF. The reaction is kept under argon for the entire time. After 10
min, methyl iodide (1.1 equiv., 0.39 mmol) was added and remaining DMF (2 mL) was added. The reaction was allowed to run under argon for 4 hrs at room temperature. DMF was stripped off using high pressure. The product was then extracted using ethyl acetate and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a brown solid in 35 % yield. TLC $R_f$ 0.21 (EtOAc: Hex; 1:1), $^1$H NMR (400 MHz, DMSO-d6): $\delta$ 1.35 (s, 3H, 2CH$_3$), 2.84 (s, 2H, 2CH$_2$), 3.48 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4’OCH$_3$), 6.92-6.94 (d, 2H, $J$ = 8.8 Hz), 7.07-7.08 (d, 2H, $J$ = 8.8 Hz), Anal. calcd. for C$_{12}$H$_{14}$ClN$_5$O: C, 51.53; H, 5.04; N, 25.04; Cl, 12.67, Found C = 51.70%, H = 5.13%, N = 24.98%, Cl = 12.53 %, M.P. = 141-142 °C.

6-chloro-N5,2-diethyl-N4-(4-methoxyphenyl)-N4-methylpyrimidine-4,5-diamine (147)

Compound 146 (100 mg, 1 equiv, 0.36 mmol) was taken in a 3 necked round bottom flask covered with septums and an adapter. NaH (1.1 equiv, 0.39 mmol) was added and was diluted with 3 mL of DMF. The reaction is kept under argon for the entire time. After 10 min, methyl iodide (1.1 equiv., 0.39 mmol) was added and remaining DMF (2 mL) was added. The reaction was allowed to run under argon for 4 hrs at room temperature. DMF was stripped off using high pressure. The product was then extracted using ethyl acetate and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 147 (65 % yield as a light brown solid). TLC $R_f$ 0.30 (EtOAc: Hex; 1:1), $^1$H
NMR (400 MHz, DMSO-d6): $\delta$ 1.35 (s, 3H, 2CH$_3$), 2.84 (s, 2H, 2CH$_2$), 3.48 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4’OCH$_3$), 6.92-6.94 (d, 2H, $J$ = 8.8 Hz), 7.07-7.08 (d, 2H, $J$ = 8.8 Hz). Anal. calcd. for $C_{13}H_{16}ClN_5O$: C, 53.15; H, 5.49; N, 23.84; Cl, 12.07; Found C = 53.36%, H = 5.58%, N = 23.56%, Cl = 11.84%, M.P. = 155-156ºC.

6-chloro-N$_4$-(4-methoxyphenyl)-N$_4$-methylpyrimidine-2,4,5-triamine (148)

4,6-dichloropyrimidine-2,5-diamine (40 mg, 1 equiv, 223 µmol), 4-methoxyaniline (46 mg, 0.34 mmol, 1.5 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 120 ºC for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a dark brown solid in 72 % yield. TLC $R_f$ 0.45 (EtOAc: Hex; 1:1) $^1$H NMR (CDCl$_3$, 400MHz): 2.96 (s, 2H, 2NH$_2$), $\delta$ 3.18 (s, 3H, 4NCH$_3$), 3.84 (s, 3H, 4’OCH$_3$), 4.70 (s, 2H, 5NH$_2$), 6.90-6.91 (d, C’2, 6 aromatic), 7.06-7.07 (d, C’3, 5). Anal. calcd. for $C_{14}H_{18}ClN_5O$: C, 54.63; H, 5.90; N, 22.75; Cl, 11.52; Found C = 54.83%, H = 5.86%, N = 22.55%, Cl = 11.36 %, M.P. = 172-174 ºC.

6-chloro-N$_4$-(4-methoxyphenyl)-N$_4$N$_5$-dimethylpyrimidine-2,4,5-triamine (149)

Compound 148 (100 mg, 1 equiv, 0.36 mmol) was taken in a 3 necked round bottom flask covered with septums, and an adapter. NaH (1.1 equiv, 0.39 mmol) was added and was diluted with 3 mL of DMF. The reaction is kept under argon for the entire time. After 10 min, methyl iodide (1.1 equiv, 0.39 mmol) was added and remaining DMF (2 mL) was
added. The reaction was allowed to run under argon for 4 hrs at room temperature. DMF was stripped off using high pressure. The product was then extracted using ethyl acetate and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 22 % yield. TLC $R_f$ 0.6 (EtOAc: Hex; 1:1) $^1$H NMR (400 MHz, DMSO-d6): 2.92 (s, 2H, 2NH$_2$), 2.97 (s, 3H, 4NCH$_3$), 3.40 (s, 3H, 4’OCH$_3$), 3.83 (s, 3H, 5NCH$_3$), 4.84 (s, 2H, 5NH), 6.90-6.91 (d, C’2, 6 aromatic), 7.06-7.07 (d, C’3, 5). Anal. calcd. for C$_{13}$H$_{16}$ClN$_5$O: C, 53.15; H, 5.49; N, 23.84; Cl, 12.07; Found C = 53.36%, H = 5.58%, N = 23.56%, Cl = 11.84 %, M.P. = 148-150 ºC.

6-chloro-N5-ethyl-N4-(4-methoxyphenyl)-N4-methylpyrimidine-2,4,5-triamine (150)

Compound 148 (100 mg, 1 equiv, 0.36 mmol) was taken in a 3 necked round bottom flask covered with septums, and an adapter. NaH (1.1 equiv, 0.39 mmol) was added and was diluted with 3 mL of DMF. The reaction is kept under argon for the entire time. After 10 min, ethyl iodide (1.1 equiv., 0.39 mmol) was added and remaining DMF (2 mL) was added. The reaction was allowed to run under argon for 4 hrs at room temperature. DMF was stripped off using high pressure. The product was then extracted using ethyl acetate and dried under sodium sulfate. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a dark brown solid in 53 % yield. TLC $R_f$ 0.6 (EtOAc: Hex; 1:1), $^1$H NMR (400 MHz, DMSO-d6): 2.92 (s, 2H, 2NH$_2$), 2.97 (s, 3H, 4NCH$_3$), 3.40 (s, 3H, 4’OCH$_3$), 3.83 (s, 3H, 5NCH$_3$), 4.84 (s, 2H, 5NH), 6.90-6.91 (d, C’2, 6 aromatic), 7.06-7.07 (d, C’3,
5), Anal. calcd. for C_{14}H_{18}ClN_{5}O: C, 54.63; H, 5.90; N, 22.75; Cl, 11.52, Found C = 54.83%, H = 5.86%, N = 22.55%, Cl = 11.36 %, M.P. = 90-92 ºC.

6-chloro-N4,2-dimethyl-N4-(4-(methylthio)phenyl)pyrimidine-4,5-diamine (151)

2-methyl-4,6 dichloro-5-aminopyrimidine (1 g, 5.44 mmol), N-methyl-4-(methylthio)aniline (1.1 g, 7.18 mmol, 1.2 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 120 ºC for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a light brown solid in 73 % yield. TLC $R_f$ 0.80 (EtOAc: Hex; 1:1) $^1$H NMR (DMSO-$d_6$, 400MHz): δ 2.39 (s, 3H, CH$_3$), 2.46 (s, 3H, 4NCH$_3$), 3.34 (s, 3H, 4'OCH$_3$), 4.32 (s, 2H, 5NH$_2$), 6.92-6.94 (d, C’2, 6 aromatic), 7.22-7.25(d, C’3, 5). Anal. calcd. for C$_{13}$H$_{15}$ClN$_4$S. 0.2C$_6$H$_{14}$: C, 54.17; H, 5.21; N, 18.41; Cl, 11.66; S, 10.54, Found C = 54.54%, H = 5.41%, N = 18.08%, Cl = 11.34 %, S = 10.39 %, M.P. = 93-94 ºC.

4-chloro-6-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methylpyrimidin-5-amine (152)

2-methyl-4,6-dichloro-5-aminopyrimidine (1 g, 5.62 mmol), 6-methoxy-1,2,3,4-tetrahydroquinoline (1.1 g, 6.74 mmol, 1.2 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 120 ºC for 2 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column
chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a solid in 20% yield. TLC Rf 0.67 (EtOAc: Hex; 1:1); 1H NMR (DMSO-d6, 400MHz): δ 1.91-1.94 (t, 2H, CH2), 2.35 (s, 3H, CH3), 2.76-2.79 (t, 2H, CH2), 3.61-3.64 (t, 2H, CH2), 3.70 (s, 3H, 4’OCH3), 4.61 (s, 2H, 5NH2), 6.28-6.31 (d, 1H, C’8 aromatic), 6.60-6.63(dd, 1H, C’7), 6.74 (s, 1H, C’5), Anal. calcd. for C15H17ClN4O: C, 59.11; H, 5.62; N, 18.38; Cl, 11.63, Found C = 58.82%, H = 5.59%, N = 18.16%, Cl = 11.50 %, M.P. = 107-109 °C.

6-chloro-N4-(2,3-dihydrobenzofuran-5-yl)-N4,2-dimethylpyrimidine-4,5-diamine (153)

2-methyl-4,6-dichloro-5-aminopyrimdine (100 mg, 0.562 mmol, 1 equiv) and N-methyl-2,3-dihydrobenzofuran-5-amine (0.674 mmol, 1.2 equiv) were dissolved in dioxane (5 mL). To this solution, was added 37 % HCl (cat.). The mixture was heated at 120 ° C in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH4OH. The dioxane is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 70 mg (42 %) as a light brown solid: TLC Rf = 0.45 (EtOAc: Hex, 1:1); M; 1H NMR (400 MHz, DMSO-d6): δ 2.38 (s, 3H), 3.14-3.19 (t, J = 8.7 Hz, 2H), 3.28 (s, 3H), 3.98 (s, 2H), 4.52-4.56 (t, J = 8.7 Hz, 2H), 6.73-6.75 (d, J = 8.4 Hz, 1H), 6.82-6.84 (dd, J = 2.3 Hz, J = 8.4 Hz, 1H), 7.06 (s, 1H). Anal. calcd. for C14H15ClN4O: C, 57.83; H, 5.20; N, 19.27; Cl, 12.19, Found C = 57.74%, H = 5.20%, N = 19.21%, Cl = 12.03%, M.P. = 101.5-103.7 °C.
6-chloro-N4-(4-methoxyphenyl)-2-methylpyrimidine-4,5-diamine (154)

2-methyl-4,6-dichloro-5-aminopyrimidine (2 g, 11.23 mmol, 1 equiv) and the 4-methoxy aniline (10.11 mmol, 1.25 equiv) were dissolved in butanol (15 mL). To this solution, was added 37 % HCl (cat.). The mixture was heated at 120 °C in microwave with stirring for 1.5 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of aq. NH₄OH. The butanol is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 154 (75%) as a grey solid: TLC Rf 0.42 (EtOAc: Hex, 1:1); ¹H NMR (400 MHz, DMSO-d6): δ 2.27 (s, 3H), 3.75 (s, 3H), 5.11 (s, 2H), 6.90-6.93 (d, J = 9.1 Hz, 2H), 6.59-6.62 (d, J = 9.1 Hz, 2H), 8.40 (s, 1H) (C₁₂H₁₃ClN₄O), Anal. calcd. for C₁₂H₁₃ClN₄O: C, 54.45; H, 4.95; N, 21.17; Cl, 13.39; Found C = 54.49%, H = 5.05%, N = 21.02%, Cl = 13.40%, M.P. = 170.1 °C.

6-chloro-2-methyl-N4-(4-(methylthio)phenyl)pyrimidine-4,5-diamine (155)

2-methyl-4,6-dichloro-5-aminopyrimidine (100 mg, 0.562 mmol, 1 equiv) and the 4-(methylthio)aniline (0.674 mmol, 1.2 equiv) were dissolved in butanol (15 mL). To this solution, was added 37 % HCl (cat.). The mixture was heated at 120 °C in microwave with stirring for 1.5 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of aq. NH₄OH. The butanol is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 155 (85%) as a grey solid: TLC Rf = 0.49 (EtOAc: Hex, 1:1); ¹H NMR (400 MHz, DMSO-d6): δ 2.31 (s, 3H), 2.46 (s, 3H),
5.12 (s, 2H), 7.25-7.27 (d, J = 8.8 Hz, 2H), 7.70-7.72 (d, J = 8.8 Hz, 2H), 8.53 (s, 1H),
Anal. calcd. for C_{12}H_{13}ClN_{4}S: C, 51.33%, H, 4.67%, N, 19.95%, Cl, 12.63%, S, 11.42%,
Found C = 51.26%, H = 4.67%, N = 19.80%, Cl = 12.52%, S = 11.32%, M.P. = 209-211 °C.

**N4,N6-bis(4-methoxyphenyl)-N4,N6,2-trimethylpyrimidine-4,5,6-triamine (156)**

2-methyl-4,6-dichloro-5-aminopyrimidine (1 g, 5.62 mmol), 4-methoxyaniline (1.55 g, 11.24 mmol, 2 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 160 °C for 3 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a dark brown solid in 60 % yield. TLC R_{f} 0.18 (EtOAc: Hex; 1:1) \(^{1}\)H NMR (DMSO-\text{d}_6, 400MHz): \(\delta\) 2.39 (s, 2-C 3H), 3.01 (s, NH\textsubscript{2} 2H), 3.27 (s, 4,6-N- 6H), 3.70 (s, 4'O-CH\textsubscript{3}, 6H), 6.81-6.86 (m, aromatic, 8H) Anal. calcd. for C_{21}H_{25}N_{5}O_{2}: C, 66.47; H, 6.64; N, 18.46; Found C = 66.50%, H = 6.59%, N = 18.22%, M.P. = 101-103 °C.

**N4,N6,2-trimethyl-N4,N6-bis(4-(methylthio)phenyl)pyrimidine-4,5,6-triamine (159)**

2-methyl-4,6-dichloro-5-aminopyrimidine (1 g, 5.62 mmol), 4-methoxy N-methylaniline (1.55 g, 11.24 mmol, 2 equiv) and catalytic amount of conc. HCl were added to a microwave vial in 15 mL dioxane and heated at 160 °C for 3 h. The reaction was stopped and dioxane was stripped off and reaction mixture neutralized to pH 8. Silica gel was added
and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a greyish solid in 32 % yield. TLC Rf 0.25 (EtOAc: Hex; 1:1), $^1$H NMR (DMSO- $d_6$, 400MHz): $\delta$ 2.39 (s, 2-C 3H), 2.41 (s, 4,6-N-6H), 3.32 (s, 4’-O-CH$_3$, 6H), 3.50 (s, NH$_2$ 2H), 6.73-6.78 (m, aromatic, 4H), 7.15-7.20 (m, aromatic, 4H), Analysis calculated for C$_{21}$H$_{25}$N$_5$S$_2$: C, 61.28; H, 6.12; N, 17.02; S, 15.58, Found C = 61.00%, H = 6.09%, N = 17.28%, S = 15.34%, M.P. -117-119 º C.

4,6-bis(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methylpyrimidin-5-amine (160)

2-methyl-4,6-dichloro-5-aminopyrimidine (100 mg, 0.562 mmol, 1 equiv) and the 6-methoxy-1,2,3,4-tetrahydroquinoline (0.674 mmol, 1.2 equiv) were dissolved in 1,4 dioxane (5 mL). To this solution was added 37 % HCl (2-3 drops). The mixture was heated at 150 ºC in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH$_4$OH. Solvents are then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 160 in 65% yield as an off-white solid: TLC Rf 0.56 (EtOAc: Hex, 1:1); $^1$H NMR (400 MHz, DMSO-d6): $\delta$ 1.94-1.97 (p, 4H), 2.32 (s, 3H), 2.79-2.82 (t, J = 6.5Hz, 3H), 3.59-3.62 (t, 4H), 3.69 (s, 6H), 3.73 (s, 2H), 6.25-6.28 (d, J = 8.8Hz, 2H) 6.62-6.65 (dd, J = 2.9Hz, J = 8.8Hz, 2H), 6.69-6.70 (d, J = 2.8Hz, 2H). Analysis calculated for C$_{25}$H$_{29}$N$_5$O$_2$: 0.1H$_2$O: C, 69.29; H, 6.79; N, 16.16, Found C = 69.08%, H = 6.57%, N = 16.14%, M.P. = 166.5 º C.
\( N-(2,3\text{-dihydrobenzofuran-5-yl})-N,2\text{-dimethyl-7\textit{H}}\text{-pyrrolo}[2,3-d]\text{-pyrimidin-4-amine} \) (162)

4-chloro-2-methyl-7\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and \( N\)-methyl-2,3-dihydrobenzofuran-5-amine (0.674 mmol, 1.2 equiv) were dissolved in \textit{n}-butanol (5 mL). To this solution was added 37 \% HCl (2-3 drops). The mixture was heated at 150 \textdegree C in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH\textsubscript{4}OH. Solvents are then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 162 in 65\% yield as an off-white solid: TLC R\text{f} 0.90 (EtOAc: Hex, 1:1; \textsuperscript{1}H NMR (500 MHz, DMSO-d\textsubscript{6}): \( \delta \)) 2.44 (s, 3H), 3.18-3.20 (t, J = 8.4 Hz, 2H), 3.43 (s, 3H), 4.58-4.60 (t, J = 8.4 Hz, 2H), 4.58 (s, 1H), 6.73 (s, 1H), 6.81-6.83 (d, J = 8.2 Hz, 1H), 7.01-7.03 (d, J = 7.9 Hz, 1H), 7.20 (s, 1H), 11.26 (s, 1H). Analysis calculated for C\textsubscript{16}H\textsubscript{16}N\textsubscript{4}O: C, 68.55; H, 5.75; N, 19.99, Found C = 68.34\%, H = 5.73\%, N = 19.88\%, M.P. = 243-245 \textdegree C.

\( N-(5\text{-methoxypyridin-2-yl})-N,2\text{-dimethyl-7\textit{H}}\text{-pyrrolo}[2,3-d]\text{-pyrimidin-4-amine} \) (163)

4-chloro-2-methyl-7\textit{H}-pyrrolo[2,3-\textit{d}]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and 5-methoxy-\( N\)-methylpyridin-2-amine (0.674 mmol, 1.2 equiv) were dissolved in butanol. To this solution was added 37 \% HCl (2-3 drops). The mixture was heated at 110 \textdegree C with stirring for 5 h. Neutralization was done by the addition of NH\textsubscript{4}OH. Solvents are then stripped off. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl\textsubscript{3} followed by 3\% MeOH in CHCl\textsubscript{3} and then 5\% MeOH in CHCl\textsubscript{3}. Fractions
containing the product were pooled and evaporated to afford **163** in 42% yield as a brown solid: TLC R_f 0.75 (chloroform/methanol: 5:1); ^1^H NMR (400 MHz, DMSO-d6): δ 2.49 (s, 3H), δ 3.52 (s, 3H), δ 3.88 (s, 3H), 4.75 (s, 1H), 6.88 (s, 1H), 7.30 (d, J = 8.7 Hz, 1H), 7.49 (dd, J = 2.8 Hz, J = 8.7 Hz, 1H), 8.22 (d, J = 2.6Hz, 1H), 11.45 (s, 1H), Analysis calculated for C_{14}H_{15}N_{5}O. 0.25H_{2}O: C, 61.41; H, 5.71; N, 25.58, Found C = 61.38%, H = 5.65%, N = 25.53%, M.P. = 217 - 219 ºC.

**N-(5-methoxynaphthalen-1-yl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (164)**

4-chloro-2-methyl-7H-pyrrolo[2,3-d]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and 5-methoxynaphthalen-1-amine (0.674 mmol, 1.2 equiv) were dissolved in n-butanol. To this solution was added 37 % HCl (2-3 drops). The mixture was heated at 120 ºC in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH_4OH. Solvents are then stripped off. To the reaction mixture was added silica gel (1 g), and the solvent was evaporated reduced pressure. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl_3 followed by 3% MeOH in CHCl_3 and then 5% MeOH in CHCl_3. Fractions containing the product were pooled and evaporated to afford **164** in 55% yield as a grey solid: TLC R_f 0.50 (chloroform/methanol: 5:1); ^1^H NMR (400 MHz, DMSO-d6): 2.50 (s, 3H), 3.60 (s, 3H), 4.01 (s, 3H), 6.53-6.54 (m, 1H), 7.00-7.20 (d, J = 7.6Hz, 1H), 7.25-7.27 (d, J = 8.4Hz, 1H), 7.38-7.42 (m, 1H), 7.57-7.67(m, 2H), 8.29 (dd, J = 1.7Hz, J = 7.9Hz, 1H), 11.23 (s, 1H) Analysis calculated for C_{18}H_{16}N_{4}O .0.105 H_{2}O: C, 70.59; H, 5.34; N, 18.30, Found C = 70.59%, H = 5.36%, N = 18.33%, M.P. = 281-283 ºC.
5-((2-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino)naphthalen-1-ol (165)

4-chloro-2-methyl-7H-pyrrolo[2,3-d]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and 5-aminonaphthalen-1-ol (0.674 mmol, 1.2 equiv) were dissolved in dioxane. To this solution was added 37 % HCl (2-3 drops). The mixture was heated at 140 ºC in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH$_4$OH. Solvents are then stripped off. To the reaction mixture was added silica gel (1 g), and the solvent was evaporated reduced pressure. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl$_3$ followed by 3% MeOH in CHCl$_3$ and then 5% MeOH in CHCl$_3$. Fractions containing the product were pooled and evaporated to afford 165 in 51% yield as a dark brown solid: TLC R$_f$ 0.30 (chloroform/methanol: 5:1); $^1$H NMR (400 MHz, DMSO-d$_6$): δ 2.35 (s, 3H), δ 5.90 (s, 1H), 6.88-6.90 (d, J = 7.3 Hz, 1H), 6.94 (s, 1H), 7.26-7.30 (t, J = 7.9 Hz, 1H), 7.45-7.49 (t, 2H), 7.60-7.61 (d, Hz, 1H), 8.06-8.08 (d, J = 8.3 Hz, 1H), 9.27 (s, 1H), 10.19 (s, 1H), 11.39 (s, 1H). Analysis calculated for C$_{17}$H$_{14}$N$_4$O. 0.3H$_2$O: C, 69.04; H, 4.98; N, 18.95, Found C = 68.67%, H = 5.13%, N = 18.61%, M.P. = 293-295 ºC.

$N$-(5-methoxynaphthalen-2-yl)-$N,2$-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (166)

4-chloro-2-methyl-7H-pyrrolo[2,3-d]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and 5-methoxy-$N$-methylnaphthalen-2-amine (0.674 mmol, 1.2 equiv) were dissolved in $n$-butanol. To this solution was added 37 % HCl (2-3 drops). The mixture was heated at 120 ºC in microwave with stirring for 2 h. Then the reaction mixture was cooled to room
temperature. Neutralization was done by the addition of NH₄OH. Solvents are then stripped off. To the reaction mixture was added silica gel (1 g), and the solvent was evaporated reduced pressure. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃. Fractions containing the product were pooled and evaporated to afford 166 in 65% yield as a grey solid: TLC Rₚ 0.55 (chloroform/methanol: 5:1); ¹H NMR (400 MHz, DMSO-d₆): 2.50 (s, 3H), 3.60 (s, 3H), 4.01 (s, 3H), 4.45-4.46 (s, 1H), 6.69-7.00 (d, J = 7.6 Hz, 1H), 7.00-7.02 (d, J = 8.4 Hz, 1H), 7.42-7.46 (m, 3H), 7.85-7.86 (m, 1H), 8.20 (dd, J = 1.7 Hz, J = 7.9 Hz, 1H), 11.35 (s, 1H), Analysis calculated for C₁₉H₁₈N₄O. 0.2H₂O: C, 70.87; H, 5.76; N, 17.40. Found C = 70.94%, H = 5.78%, N = 17.40%, M.P. = 280-282 °C.

*N,2-dimethyl-N-(4-(methylthio)phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (167)*

4-Chloro-2-methyl-7H-pyrrolo[2,3-d]pyrimidine (100 mg, 0.562 mmol, 1 equiv) and N-methyl-4-(methylthio)aniline (0.674 mmol, 1.2 equiv) were dissolved in n-butanol (5 mL). To this solution was added 37 % HCl (2-3 drops). The mixture was heated at 120 °C in microwave with stirring for 2 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of NH₄OH. Solvents were then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 167 in 62% yield as a white solid: TLC Rₚ 0.45 (EtOAc:Hex, 1:1); ¹H NMR (400 MHz, DMSO-d₆): δ 2.47 (s, 3H), 2.53 (s, 3H), 3.48 (s, 3H), 4.64-4.66 (dd, J = 2 Hz, J = 3.5 Hz, 1H), 6.78-6.80 (dd, J = 2.4 Hz, J = 3.5 Hz, 1H), 7.25-7.27 (d, 2H, J = 8.8 Hz), 7.32-7.35 (d, 2H, J = 8.8 Hz), 11.34
(s, 1H). Analysis calculated for C$_{15}$H$_{16}$N$_4$S:  C, 63.35; H, 5.67; N, 19.70; S, 11.27, Found C = 63.44%, H = 5.64%, N = 19.71%, S = 11.28%, M.P. = 155 °C.

2-(tert-butyl)-N-(4-methoxyphenyl)-N$_5$-dimethyloxazolo[5,4-d]pyrimidin-7-amine (171)

Compound 122 (50 mg, 1 equiv, 0.18 mmol) was dissolved in pivalic anhydride (6 mL) and the reaction was run at 150 °C for 12 h. The solvent was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded a grey solid in 60 % yield. TLC $R_f$ 0.25 (EtOAc: Hex; 1:1); $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.34 (s, 9H, CH$_3$), 2.55 (s, 3H, 2CH$_3$), 3.77 (s, 3H, 4NCH$_3$), 3.87 (s, 3H, 4’OCH$_3$), 6.94-6.96 (d,2H, J = 8.8 Hz), 7.22-7.25 (d,2H, J = 8.8 Hz). Analysis calculated for C$_{18}$H$_{24}$N$_4$O$_2$:  C, 65.83; H, 7.26; N, 17.06; Found C = 65.96%, H = 6.88, N = 17.08%, M.P. = 120-122 °C.

N$_4$N$_9$-bis(4-methoxyphenyl)-N$_4$N$_9$2,7-tetramethylpyrimido[4,5-g]pteridine-4,9-diamine (172)

Compound 122 (500 mg, 1.79 mmol) was taken and added to benzylpotassium trifluoroobate (355 mg, 1.79 mmol, 1 equiv) in a microwave vial and cesium carbonate (2 equiv) was added in addition to tetrakistriphenylphosphine palladium (0.05 equiv) in acetonitrile. The reaction was run for 3 hrs at 120 °C and after the reaction was done, acetonitrile was stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2)
afforded a red solid in 15 % yield. TLC $R_f$ 0.25 (EtOAc: Hex; 1:1); $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 2.59 (s, 6H, 2CH$_3$), 3.89 (s, 6H, 4NCH$_3$), 3.98 (s, 6H, 4’OCH$_3$), 6.96-6.98 (d, 4H, J = 8.8 Hz), 7.19-7.21 (d, 4H, J = 8.8 Hz). Analysis calculated for C$_{26}$H$_{26}$N$_8$O$_2$. 0.2CH$_3$COOC$_2$H$_5$: C, 64.36; H, 5.56; N, 22.40; Found C = 64.47%, H = 5.54%, N = 22.45%, M.P. = 243-245 ºC.

$N_4$-(2-bromo-4-methoxyphenyl)-6-chloro-2-methylpyrimidine-4,5-diamine (241)

2-Methyl-4,6-dichloropyrimidine (2 g, 11.23 mmol, 1 equiv) and the 2-bromo-4-methoxy aniline (10.11 mmol, 1.25 equiv) were dissolved in butanol (15 mL). To this solution, was added 37 % HCl (cat.). The mixture was heated at 120 ºC in microwave with stirring for 1.5 h. Then the reaction mixture was cooled to room temperature. Neutralization was done by the addition of aq. NH$_4$OH. The butanol is then stripped off. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 241 (79%) as a grey solid: TLC $R_f$ 0.55 (EtOAc:Hex, 1:1); $^1$H NMR (400 MHz, DMSO-d6): $\delta$ 2.17 (s, 3H), 3.79 (s, 3H), 5.11 (s, 2H), 6.97-7.01 (dd, 1H), 7.25-7.26 (d, J = 9.1 Hz, 2H), 7.41-7.43 (d, J = 9.1 Hz, 2H) Anal. calcd. for C$_{12}$H$_{12}$BrClN$_4$O: C, 41.95; H, 3.52; N, 16.31; Cl, 10.32; Br, 23.25, Found C = 42.08%, H = 3.58%, N = 16.15%, Cl = 10.18%, Br = 23.11%, M.P. = 135-136 ºC.

$N$-methyl-4-(methylthio)aniline (193)

Aniline 192 (5 g, 1 equiv) was added to a suspension of NaOMe (5 equiv) in MeOH (8-15 mL). The resulting solution was poured into a suspension of paraformaldehyde (1.4 equiv.) in MeOH (5-10 mL). The reaction was stirred at rt for 4.5 h and then sodium borohydride
(1.2 equiv.) was added and solution heated to reflux for 2 h. The reaction mixture was allowed to cool to room temperature and then filtered with methanol washings. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 4.90 g (90%) as a dark red liquid: TLC Rf 0.80 (EtOAc:Hex, 1:1); δ 1H NMR (400 MHz, DMSO-d6) δ 2.34 (s, 3H), 2.64-2.66 (d, J = 5.1 Hz, 3H), 5.72-5.73 (q, 1H), 6.50-6.52 (d, J = 8.6 Hz, 2H), 7.11-7.13 (d, J = 8.6 Hz, 2H). 1H NMR matches prior synthesis.248

**N-methyl-2,3-dihydrobenzofuran-5-amine (196)**

Aniline 195 (1 g, 1 equiv) was added to a suspension of NaOMe (5 equiv.) in MeOH (8-15 mL). The resulting solution was poured into a suspension of paraformaldehyde (1.4 equiv.) in MeOH (5-10 mL). The reaction was stirred at rt for 5 h and then sodium borohydride (1.2 equiv.) was added and solution heated to reflux for 2 h. The reaction mixture was allowed to cool to room temp. and then filtered with methanol washings. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 0.93 g (85%) as a brown liquid: TLC Rf 0.90 (EtOAc:Hex, 1:1); 1H NMR (DMSO-d6) δ 1H NMR (400 MHz, DMSO-d6) δ 2.61 (s, 3H), 3.04-3.08 (t, J = 8.5 Hz, 2H), 4.36-4.40 (t, J = 8.6 Hz, 2H), 5.09 (s, 1H), 6.25-6.28 (dd, J = 2.4 Hz, J = 8.4 Hz, 1H), 6.48-6.49 (s, 1H), 6.51-6.54 (d, J = 8.5 Hz, 1H). The reaction was carried on without additional characterization.
5-methoxy-N-methylnaphthalen-1-amine (200)

Aniline 199 (5 g, 1 equiv) was added to a suspension of NaOMe (5 equiv) in MeOH (8-15 mL). The resulting solution was poured into a suspension of paraformaldehyde (1.4 equiv.) in MeOH (5-10 mL). The reaction was stirred at rt for 4.5 h and then sodium borohydride (1.2 equiv.) was added and solution heated to reflux for 2 h. The reaction mixture was allowed to cool to room temperature and then filtered with methanol washings. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded (75%) as a grey solid: TLC R_f 0.15 (EtOAc:Hex, 1:1); ¹H NMR (400 MHz, DMSO-d6): δ ¹H NMR (400 MHz, DMSO) 2.84 (d, J=4.8Hz, 3H), 3.93 (s, 3H), 6.23-6.25 (q, J = 4.5Hz, 1H), 6.44-6.46 (d, J = 7.1Hz, 1H), 6.90-6.92 (d, J = 7.6Hz, 1H), 7.26-7.33 (m, 2H), 7.37-7.39 (d, 1H), 7.64-7.66 (d, J = 8.6Hz, 1H), M.P. = 195 °C. The reaction was carried on without additional characterization.

Ethyl 4-hydroxybenzoate (207a)

4-Hydroxybenzoic acid (1.37 g, 10 mmol) was dissolved in a minimum amount of absolute ethanol (15 mL). As 1 mL of concentrated sulfuric acid was added, a white suspension formed which was vigorously stirred and refluxed for 3 h to give a clear solution. The reaction mixture was cooled to 40 °C and poured into a saturated solution of sodium bicarbonate in water (100 mL). Vigorous effervescence was observed with the precipitation of a white solid. The precipitate was filtered and dried in air at room temperature to afford 207a (62%) as a white solid: TLC R_f = 0.80 (EtOAc:Hex, 1:1); ¹H NMR (400 MHz,
DMSO-d6): δ 1.31 (t, 3H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H). M.P. = 131 °C. M.P. matches prior synthesis.249

**Ethyl 4-((6-hydroxyhexyl)oxy)benzoate (208a)**

A mixture of 207a (0.3 g, 3.2 mmol), K₂CO₃ (1.3 g, 9.6 mmol) and 6-bromo-hexanol (0.34 ml, 4.8 mmol) in MeCN (25 mL) was heated for 6 h at 90 °C. The mixture was cooled to room temperature and then H₂O was added and the mixture was diluted with Et₂O. The organic layer was separated, and the aqueous layer was extracted with Et₂O. The combined organic layers were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 208a (45%) as a liquid: TLC Rf 0.50 (EtOAc:Hex, 1:1); ¹H NMR (400 MHz, DMSO-d6): δ 1.31 (t, 3H), 1.41 (m, 2H), 1.58 (m, 2H), 1.73 (m, 2H), 2.46 (m, 2H), 3.81 (m, 2H), 4.03 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H). Rf matches prior synthesis.250 The reaction was carried on without additional characterization.

**Ethyl 4-((6-oxohexyl)oxy)benzoate (209a)**

To a solution of DMP (3.7 g, 14.4 mmol) in CH₂Cl₂ (14 mL) was added a solution of 208a (1.85 g, 12 mmol) in CH₂Cl₂ (60 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 hexanes and ethyl acetate (10:1 to 1:2) afforded aldehyde 209a (1.64 g, 10.8 mmol) in 90% yield as a transparent oil. TLC $R_f=0.40$ (10% EtOAc/hexanes). Fracctions containing the product were pooled and evaporated to afford (75%) as a liquid: TLC $R_f$ 0.70 (EtOAc:Hex, 1:1); $^1$H NMR (400 MHz, DMSO-d6): δ 1.31 (t, 3H), 1.41 (m, 2H), 1.58 (m, 2H), 1.73 (m, 2H), 2.46 (m, 2H), 3.81 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, $J = 1.9$ Hz, 2H), 7.84-7.86 (d, $J = 1.9$ Hz, 2H), 9.67 (s, 1H). The reaction was carried on without additional characterization.

**Ethyl-4-(4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butoxy)benzoate (211a)**

To a solution of bromine (0.1 mL)–dioxane (0.3 mL) solution, 1 mL DCM was added. The viscous solution was stirred for 10 min at 0 °C, and then a solution of 209a (5.00 g, 37.26 mmol) in DCM was added dropwise to it at 0 °C. The mixture was stirred at 25°C for 1 h. After 1 h, the mixture was poured into water and extracted with DCM. The combined organic layers were washed with brine, dried (Na$_2$SO$_4$) and evaporated to give the crude title product, which was used directly in the next step reaction. To a suspension of 2,4-diamino-6-hydroxypyrimidine (4.7 g, 37.26 mmol) and sodium acetate (6.11 g, 74.53 mmol) in water was added methyl 4-((5-bromo-6-oxohexyl)oxy)benzoate (210a, 7.94 g, 37.26 mmol) in DMF. The reaction mixture was stirred at 40 °C for 12 h. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 211a. (20%) as a solid: TLC $R_f$ 0.65 (chloroform: methanol, 5:1); $^1$H NMR (400 MHz, DMSO-d6): δ 1.31 (t, 3H), 1.61 (m, 2H),...
1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58(s, IH), 10.3 (s, 2H), M.P. = 220-222 ºC. The reaction was carried on without additional characterization.

4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoic acid (212a)

The ethyl ester of 211a (0.5 g, 0.37 mmol was hydrolyzed using 5 mL 1N sodium hydroxide solution at room temperature for 5 hours to produce acid 212a. The pH of the reaction mixture was reduced to 2 (acidic) using 1N HCl solution. The precipitates formed were filtered and dried to yield 212a (88%) as a solid: TLC Rf 0.65 (chloroform: methanol, 5:1); \(^1\)H NMR (DMSO-d6) \(\delta\) 1.61 (m, 2H), 1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58(s, IH), 12.5 (s, 2H), M.P. = 225-227 ºC. The reaction was carried on without additional characterization.

Diethyl(4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoyl)-L-glutamate (213a)

The acid 212a was coupled with commercially available L-glutamate-diethylester in the presence of the base NMM, and CDMT to give coupled product 213a. The solvents were stripped off, the silica was added and the resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl\(_3\) followed by 3% MeOH in CHCl\(_3\) and then 5% MeOH in CHCl\(_3\). Fractions containing the product were pooled and evaporated to afford 213a (85%) as a solid: TLC Rf = 0.5 (chloroform: methanol, 5:1); \(^1\)H NMR (400 MHz, DMSO-
d(6): δ 1.2 (1.61 (m, 2H), 1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 4.0-4.01 (m, 4H), 4.39-4.41 (m, 1H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58(s, 1H), 12.5 (s, 2H), Decomposes above 250 ºC. The reaction was carried on without additional characterization.

(4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoyl)-L-glutamic acid (173)

The ethyl ester of 213a was hydrolyzed using 1 N sodium hydroxide solution at room temperature for 5 hours to produce acid 173. The pH of the reaction mixture was reduced to pH = 2 (acidic) using 1N HCl solution. The precipitates formed were filtered and dried to yield final compound 173 (75%) as a solid: TLC Rf 0.5 (chloroform: methanol, 5:1); 1H NMR (DMSO-d6) δ 1.70-1.80 (m, 4H, CH2), 1.95-1.97 (m, 1H, CH2), 2.07-2.09 (m, 1H, CH2), 2.35 (m, 2H, CH2), 2.63 (m, 2H, CH2), 4.04 (t, 2H, OCH2), 4.39 (t, 1H, CH), 5.98 (d, 2H, 2-NH2, exch), 6.38 (s, 1H, 6-CH), 6.98-7.00 (d, 2H, Ar-CH, J = 8 Hz), 7.84–7.86 (d, 2H, Ar-CH, J = 8 Hz), 8.44-8.46 (d, 1H, Ar-CONH, J = 8 Hz, exch), 10.12 (s, 1H, 3-NH, exch), 10.65 (s, 1H, 7-NH, exch), 12.38-12.41 (s, 2H, COOH, exch). Analysis calculated for C22H25N3O7: 1.43H2O: C, 53.14%; H, 5.65%; N, 14.08%; Found C = 53.22%, H = 5.37%, N = 13.69%, decomposes above 250 ºC.

Ethyl 4-mercaptobenzoate (207b)

4-Mercaptobenzoic acid (1.1 g, 10 mmol was dissolved in a minimum amount of absolute ethanol (15 mL). As 1 mL of concentrated sulfuric acid was added, a white suspension
formed which was vigorously stirred and refluxed for 3 h to give a clear solution. The reaction mixture was cooled to 40 °C and poured into a saturated solution of sodium bicarbonate in water (100 mL). Vigorous effervescence was observed with the precipitation of a white solid. The precipitate was filtered and dried in air at room temperature to afford a white solid in 62 % yield. TLC Rf 0.6 (EtOAc: Hex; 1:1) TLC Rf 0.6 (CH3OH: CHCl3; 1:10) 1H NMR (400 MHz, DMSO-d6) δ 7.79-7.81 (2H, d), δ 7.41-7.43 (2H, d), 5.99 (1H, s), 4.29 (2H, q), 1.30 (3H, t). M.P. = 220-221 °C. 1H NMR matches prior synthesis. 253

Ethyl 4-((6-hydroxyhexyl)thio)benzoate (208b) 254

A mixture of 207a (0.3 g, 3.2 mmol), Cs2CO3 (1.3 g, 9.6 mmol) and 6-bromo-hexanol (0.34 mL, 4.8 mmol) in DMF (25 mL) was heated for 6 h at 90 °C. The mixture was cooled to room temperature and then H2O was added and the mixture was diluted with Et2O. The organic layer was separated, and the aqueous layer was extracted with Et2O. The combined organic layers were washed with brine, dried over MgSO4 and concentrated under reduced pressure. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded 208b (75%) as a liquid: TLC Rf 0.50 (EtOAc:Hex, 1:1); 1H NMR (DMSO-d6) δ 1.31 (t, 3H), 1.41 (m, 2H), 1.58 (m, 2H), 1.73 (m, 2H), 2.46 (m, 2H), 3.81 (m, 2H), 4.03 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H). The reaction was carried on without additional characterization.
**Ethyl 4-((6-oxohexyl)thio)benzoate (209b)**

To a solution of DMP (3.7 g, 14.4 mmol) in CH$_2$Cl$_2$ (14 mL) was added a solution of 208b (1.85 g, 12 mmol) in CH$_2$Cl$_2$ (60 mL) at 0 °C, and the mixture was stirred for 4 hours at room temperature. The aldehyde product was extracted with organic solvent CH$_2$Cl$_2$, and the combined extracts were washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. Silica gel was added and the solvent evaporated to obtain a plug. Purification by column chromatography using hexanes and ethyl acetate (10:1 to 1:2) afforded aldehyde 209b (1.64 g, 10.8 mmol) in 65% yield as a transparent oil. TLC Rf=0.40 (10% EtOAc:Hex).$^{251}$ (75%) as a liquid: TLC Rf 0.70 (EtOAc:Hex, 1:1); $^1$H NMR (400 MHz, DMSO-d$_6$): δ 1.31 (t, 3H), 1.41 (m, 2H), 1.58 (m, 2H), 1.73 (m, 2H), 2.46 (m, 2H), 3.81 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 9.67 (s, IH). The reaction was carried on without additional characterization.

**Ethyl 4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-l)butyl)thio)benzoate (211b)** $^{252}$

To a solution of bromine (0.1 mL)–dioxane (0.3 mL) solution, 1 mL DCM was added. The viscous solution was stirred for 10 min at 0 °C, and then a solution of 209b in DCM was added dropwise to it (5.00 g, 37.26 mmol) at 0 °C. The mixture was stirred at 25 °C for 1 h. After 1 h, the mixture was poured into water and extracted with DCM. The combined organic layers were washed with brine, dried (Na$_2$SO$_4$) and evaporated to give the crude title product, which was used directly in
the next step reaction. To a suspension of 2,4-diamino-6-hydroxypyrimidine (4.7 g, 37.26 mmol) and sodium acetate (6.11 g, 74.53 mmol) in water was added ethyl 4-((5-bromo-6-oxohexyl)thio)benzoate (210b, 7.94 g, 37.26 mmol) in DMF The reaction mixture was stirred at 40 °C for 12 h. To the reaction mixture was added silica gel, and the solvent was evaporated under reduced pressure. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃ afforded 211b (35%) as a solid: TLC Rf 0.65 (chloroform: methanol, 5:1); ¹H NMR (400 MHz, DMSO-d₆): δ 1.31 (t, 3H), 1.61 (m, 2H), 1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 4.28 (q, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58 (s, 1H), 10.3 (s, 2H). M.P. = 241-243 ºC. The reaction was carried on without additional characterization.

4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoic acid (212b)

The ethyl ester of 211b was hydrolyzed using 1N sodium hydroxide solution at room temperature for 5 hours to produce acid 212b. The pH of the reaction mixture was reduced to 2 (acidic) using 1N HCl solution. The precipitates formed were filtered and dried to yield final product (93%) as a solid: TLC Rf = 0.65 (chloroform: methanol, 5:1) ¹H NMR (400 MHz, DMSO-d₆): δ 1.61 (m, 2H), 1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58 (s, 1H), 12.5 (s, 2H). The reaction was carried on without additional characterization.
Diethyl(4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoyl)-L-glutamate (213b)

The acid 212b was coupled with L-glutamate-diethylester in the presence of the base NMM, and CDMT to give coupled product 213b. The solvents were stripped off and the plug was made using silica powder. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃. Fractions with desired Rf (TLC) were pooled and evaporated to afford 213b (65%) as a solid: TLC Rf 0.5 (chloroform: methanol, 5:1); ¹H NMR (DMSO-d6) δ 1.2 (1.61 (m, 2H), 1.72 (m, 2H), 2.6 (m, 2H), 3.1 (m, 2H), 4.0-4.01 (m, 4H), 4.39-4.41 (m, 1H), 7.38-7.40 (d, J = 1.9 Hz, 2H), 7.84-7.86 (d, J = 1.9 Hz, 2H), 8.58(s, 1H), 12.5 (s, 2H). Decomposes above 250 ºC. The reaction was carried on without additional characterization.

(4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoyl)-L-glutamic acid (174)

The ethyl ester of 213b was hydrolyzed using 1 N sodium hydroxide solution at room temperature for 5 hours to produce acid 174. The pH of the reaction mixture was reduced to 2 (acidic) using 1N HCl solution. The precipitates formed were filtered and dried to yield final 174 (92%) as a solid: TLC Rf 0.5 (chloroform: methanol, 5:1); ¹H NMR (DMSO-d6) δ 1.59-1.62 (m, 1H, CH₂), 1.73-1.76 (m, 1H, CH₂), 1.95-1.97 (m, 1H, CH₂), 2.08-2.10 (m, 1H, CH₂), 2.35 (m, 2H, CH₂), 2.63 (m, 2H, CH₂), 3.04 (t, 2H, SCH₂), 4.39 (t, 1H, CH), 6.00 (d, 2H, 2-NH₂, exch), 6.36 (s, 1H, 6-CH), 7.34-7.36 (d, 2H, Ar-CH, J = 8 Hz),
7.82–7.84 (d, 2H, Ar-CH, J = 8 Hz), 8.56–8.58 (d, 1H, Ar-CONH, J = 8 Hz, exch), 10.14 (s, 1H, 3-NH, exch), 10.65 (s, 1H, 7-NH, exch), 12.35–12.65 (s, 2H, COOH, exch).

Analysis calculated for C₂₂H₂₅N₅O₆S. 1.38H₂O:  C, 52.14; H, 5.39; N, 12.28; S, 5.62; Found C = 52.10%, H = 5.48%, N = 12.17%, S = 5.93%, calculated MW = 497.23, Decomposes above 250 °C.
VI. SUMMARY

This dissertation describes the design and synthesis of pyrimidine-based heterocycles as single agents with combination chemotherapy potential. These efforts led to the identification of structural features that are necessary for inhibition of tubulin polymerization. Structural modifications also led to the identification of antiangiogenic agents which inhibit one or more of the receptor tyrosine kinases (RTKs)-vascular endothelial growth factor receptor-2, platelet derived growth factor receptor-β and epidermal growth factor receptor. Single agents with both antiangiogenic activities as well as cytotoxicity would afford single entities that circumvent pharmacokinetic problems of multiple agents, avoid drug-drug interactions, could be used at lower doses to alleviate toxicity, devoid of overlapping toxicities, and delay or prevent tumor cell resistance. This dissertation also describes selective tumor targeting with 5-substituted pyrrolo[2,3-\(d\)]pyrimidines with heteroatom bridge substitution as GARFTase inhibitors circumventing both dose-limiting toxicity and tumor resistance associated with most prescribed antitumor agents like pemetrexed.

The novel target compounds synthesized as part of this study are listed below:

1. 6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (122)
2. 6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (123)
3. 6-chloro-N4-(4-methoxyphenyl)-N4,N5,N5,2-tetramethylpyrimidine-4,5-diamine (124)
4. 6-chloro-N5-ethyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (125)
5. 6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethyl-N5-propylpyrimidine-4,5-diamine (126)
6. N5-butyl-6-chloro-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (127)
7. 6-chloro-N5-isobutyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (128)
8. 6-benzyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (130)
9. N4-(4-methoxyphenyl)-N4,2-dimethyl-6-phenethylpyrimidine-4,5-diamine (131)
10. N4-(4-methoxyphenyl)-N4,2-dimethyl-6-(phenylethynyl)pyrimidine-4,5-diamine (132)
11. N-(4-chloro-6-((4-methoxyphenyl)(methyl)amino)-2-methylpyrimidin-5-yl)acetamide (133)
12. N-acetyl-N-(4-chloro-6-((4-methoxyphenyl)(methyl)amino)-2-methylpyrimidin-5-yl)acetamide (134)
13. N-(4-chloro-6-((4-methoxyphenyl)(methyl)amino)-2-methylpyrimidin-5-yl)pivalamide (135)
14. 6-chloro-N-(4-methoxyphenyl)-N,2-dimethyl-5-nitropyrimidin-4-amine (136)
15. N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (137)
16. N’4-(4-methoxyphenyl)-N4,2,6-trimethylpyrimidine-4,5-diamine (138)
17. 6-ethyl-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (139)
18. N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5,6-triamine (140)
19. 5-amino-6-((4-methoxyphenyl)(methyl)amino)-2-ethylpyrimidine-4-carbonitrile (141)
20. 6-iodo-N4-(4-methoxyphenyl)-N4,2-dimethylpyrimidine-4,5-diamine (142)
21. N4-(4-methoxyphenyl)-N4,2-dimethyl-6-(trifluoromethyl)pyrimidine-4,5-diamine (143)
22. 6-chloro-N4-(4-methoxyphenyl)-N4-methylpyrimidine-4,5-diamine (144)
23. 6-chloro-2-ethyl-N4-(4-methoxyphenyl)-N4-methylpyrimidine-4,5-diamine (145)
24. 6-chloro-2-ethyl-N4-(4-methoxyphenyl)-N4,N5-dimethylpyrimidine-4,5-diamine (146)
25. 6-chloro-N5,2-diethyl-N4-(4-methoxyphenyl)-N4-methylpyrimidine-4,5-diamine (147)
26. 6-chloro-N4-(4-methoxyphenyl)-N4-methylpyrimidine-2,4,5-triamine (148)
27. 6-chloro-N4-(4-methoxyphenyl)-N4,N5-dimethylpyrimidine-2,4,5-triamine (149)
28. 6-chloro-N5-ethyl-N4-(4-methoxyphenyl)-N4-methylpyrimidine-2,4,5-triamine (150)
29. 6-chloro-N4,2-dimethyl-N4-(4-(methylthio)phenyl)pyrimidine-4,5-diamine (151)
30. 4-chloro-6-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methylpyrimidin-5-amine (152)
31. 6-chloro-N4-(2,3-dihydrobenzofuran-5-yl)-N4,2-dimethylpyrimidine-4,5-diamine (153)
32. 6-chloro-N4-(4-methoxyphenyl)-2-methylpyrimidine-4,5-diamine (154)
33. 6-chloro-2-methyl-N4-(4-(methylthio)phenyl)pyrimidine-4,5-diamine (155)
34. N4,N6-bis(4-methoxyphenyl)-N4,N6,2-trimethylpyrimidine-4,5,6-triamine (156)
35. N4,N6-bis(4-methoxyphenyl)-N4,N6,2-trimethylpyrimidine-4,5,6-triamine (157)
36. N4,N6,2-trimethyl-N4,N6-bis(4-(methylthio)phenyl)pyrimidine-4,5,6-triamine (158)
37. N4,N6,2-trimethyl-N4,N6-bis(4-(methylthio)phenyl)pyrimidine-4,5,6-triamine (159)
38. 4,6-bis(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-2-methylpyrimidin-5-amine (160)
39. N-(2,3-dihydrobenzofuran-5-yl)-N2-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (162)
40. N-(5-methoxypyridin-2-yl)-N2-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (163)
41. N-(5-methoxynaphthalen-1-yl)-2-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (164)
42. 5-((2-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino)naphthalen-1-ol (165)
43. N-(5-methoxynaphthalen-2-yl)-N2-dimethyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (166)
44. N2-dimethyl-N-(4-(methylthio)phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (167)
45. N-(4-methoxyphenyl)-N2,5-trimethyloxazolo[5,4-d]pyrimidin-7-amine (170)
46. 2-(tert-butyl)-N-(4-methoxyphenyl)-N5-dimethyloxazolo[5,4-d]pyrimidin-7-amine (171)
47. N4,N9-bis(4-methoxyphenyl)-N4,N9,2,7-tetramethylpyrimido[4,5-g]pteridine-4,9-diamine (172)
48. N4-(2-bromo-4-methoxyphenyl)-6-chloro-2-methylpyrimidine-4,5-diamine
49. (4-(4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butoxy)benzoyl)-L-glutamic acid (173)

50. (4-((4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thio)benzoyl)-L-glutamic acid (174)

51. (4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethoxy)benzoyl)-L-glutamic acid (175)

52. 6-chloro-N4-(4-methoxyphenyl)-N4,N5,N5-trimethylpyrimidine-2,4,5-triamine (188)

53. 6-chloro-N4-(4-methoxyphenyl)-N2,N4,N5,N5-tetramethylpyrimidine-2,4,5-triamine (189)

7-Benzyl-pyrrolo[3,2-\textit{d}]pyrimidine 129 maintained the cytotoxic antitubulin activity of pyrrolo[3,2-\textit{d}]pyrimidine 121, but also showed improved activity against VEGFR2 and 9-fold improvement of antiangiogenic effects over 121. These observations are consistent with combination chemotherapeutic potential in a single agent.\textsuperscript{180, 199}

Compounds 130 and 131 were designed as open chain analogs of 129, to explore dual antiangiogenic and microtubule properties with the potential for multikinase inhibition. The compounds turned out to be inactive in tubulin assays. They are currently being tested for their multikinase inhibition properties. Compound 122 is an open chain conformationally flexible analog of 121. This was an intermediate in the synthesis of 130 and 131. Compound 122 showed potent activity against bovine tubulin assembly (IC\textsubscript{50} = 2.1 ± 0.04 µM), and in a VEGFR2 expressing cell line, it was determined that 122 (IC\textsubscript{50}
=33.2 nM) was 5-fold more active than 121 in this assay. Importantly, this is only 2-fold less potent than the standard sunitinib. These results are remarkable as VEGFR2 is the principal mediator of angiogenesis. Compound 122, an open chain monocyclic flexible pyrimidine displayed increased dual potency in both tubulin assays.

There could be multiple reasons why 122 is more active than pyrrolo[2,3-\textit{d}]pyrimidine 121. Electron withdrawing 6-chloro group in 122 is an obvious place to start an analysis. Any electron donating group at 6-position such as OH, NH\textsubscript{2} or an alkyl group was not tolerated. But replacement with electron withdrawing groups at the 6-position like trifluoromethyl or iodo, was tolerated. Another reason might be the size of the chloro group. However, replacement of Cl with a methyl moiety was not tolerated. A third reason might be the availability of lone pair of 5-NH\textsubscript{2} in 122. Compared to the 121, the lone pair of electrons at 5-NH\textsubscript{2} in 122 may be available for hydrogen bonding as either a hydrogen bond donor and/or acceptor. The nitrogen lone pair of compound 121 are unavailable due to conjugation. The equivalent lone pair of 122 NH\textsubscript{2} might be involved in conjugation with the pyridine ring but could still form hydrogen bonds.

So, it is likely the electron withdrawing nature of the Cl, or the hydrogen bonding acceptor properties of 5-NH\textsubscript{2} results in improved activity compared to 121.

The effect of homologation at the N5 position of 122 on tubulin and RTK activity was examined. Optimum chain length of the N5-substituent was determined. Compound 125 was found to be 18 nM (IC\textsubscript{50}) in β-III overexpressing HeLa cells, and 22.2 nM (IC\textsubscript{50}) in P-gp overexpressing NC/DR-Res cells. Increased lipophilicity of 125 may also permit better penetration across cell membranes, and produce better cellular activity. Compound 125
displayed more effective interactions with Ala316, Val315, Val181 in the tubulin site, Leu840 and Leu1035 in the VEGFR site, Ala719 in the EGFR site, and Val665 and Phe845 in the PDGFR homology model.

Compound 125 displayed combination chemotherapeutic properties in a single agent. The next goals could examine changes at other positions keeping the 6-Cl substitution unchanged. Substituted anilines could be introduced at the 4-position of the pyrimidine ring of 125 replacing the 4´-OMe aniline. The 4´-thiomethyl group was the best tolerated, and so should be examined.

Compound 125 could also be substituted with other halogens. Exploration of other electron withdrawing groups at other positions could have favorable effects in the biological assays examined.
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APPENDIX-1

The biological evaluations of were performed by Dr. Michael Ihnat (Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, Oklahoma City, OK 73117), Dr. Ernest Hamel (Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, Frederick National Laboratory for Cancer Research, National Cancer Institute, Frederick, MD 21702), Dr. Susan Mooberry (Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229), National Cancer Institute (Developmental Therapeutics Program) and Luceome Biotechnologies (1775 S. Pantano Rd, Suite 100, Tucson, AZ 85710)

Biological evaluations

The EC\textsubscript{50} (concentration required to cause 50% loss of cellular microtubules) was determined in A-10 cells\textsuperscript{1}. The effects of the compounds on interphase and mitotic microtubules were evaluated using indirect immunofluorescence techniques, and the EC\textsubscript{50} values were calculated from a minimum of three experiments.

Antiproliferative effects were evaluated against the drug sensitive MDA-MB-435 melanoma cells using sulforhodamine B assay and the IC\textsubscript{50} values (concentration required to cause 50% inhibition of proliferation) were calculated.

In experiments with purified tubulin, the control compound was combretastatin A-4 (CSA4), a potent colchicine site agent generously supplied by Dr. G. R. Pettit, Arizona State University, Tempe AZ. Tubulin polymerization was measured by turbidimetry at 350
nm in Beckman DU7400 and DU7500 recording spectrophotometers equipped with temperature controllers. In brief, 10 µM bovine brain tubulin, purified as described previously, was preincubated for 15 min in a 0.24 mL volume at 30°C containing 0.75 M monosodium glutamate (adjusted to pH 6.6 with HCl in a 2 M stock solution), varying compound concentrations, and 4% (v/v) dimethyl sulfoxide (compound solvent). Following the preincubation, which permits detection of activity in slow binding compounds such as colchicinoids, samples were chilled on ice, and 10 µL of 10 mM GTP was added (0.4 mM). The addition of GTP is an absolute requirement for assembly under these reaction conditions. All concentrations refer to the final 0.25 mL reaction volume. Samples were transferred to cuvettes held at 0°C in the recording spectrophotometers, and the temperature was jumped to 30°C, which takes less than a minute. Assembly at 30°C was followed for 30 min, and the compound concentration to inhibit extent of assembly after 30 min was determined by interpolation of data obtained with individual compound concentrations. After determining the likely range for the IC₅₀ value, 2-4 individual determinations were made, and the average from these determinations are presented in Table 1.

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.

Table 1. Effects in cellular assays and on purified tubulin

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<th>Compd.</th>
<th>Lab Record book no.</th>
<th>EC₅₀ Microtubule Depolymerization (nM)</th>
<th>MDA-MB-435 IC₅₀±SD (nM)</th>
<th>Inhibition of</th>
<th>% Colchicine Binding inhibited at 5µM compound concentration</th>
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<td>Tubulin Assembly</td>
<td>IC₅₀±SD (µM)</td>
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<td>-</td>
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Antibodies

The PY-HRP antibody was from BD Transduction Laboratories (Franklin Lakes, NJ). Antibodies against VEGFR-2 were purchased from Cell Signaling Technology (Danvers, MA).

Phosphotyrosine ELISA

Cells used were tumor cell lines naturally expressing high levels of VEGFR-2 (U251). Expression levels at the RNA level were derived from the NCI Developmental Therapeutics Program (NCI-DTP) web site public molecular target information. Briefly, cells at 60–75% confluence are placed in serum-free medium for 18 h to reduce the background phosphorylation. Cells were always >98% viable by trypan blue exclusion. Cells were then pretreated for 60 min with a dose-response relation of 100-1.4 µM compound followed in ⅓ Log increments by 100 ng/mL VEGF for 10 min. The reaction was stopped and cells permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05% Triton X-100, protease inhibitor cocktail and tyrosine phosphatase inhibitor cocktail. The TBS solution was then removed and cells fixed to the plate for 30 min at 60 °C with a further incubation in 70% ethanol for an additional 30 min. Cells were exposed to a blocking solution (TBS with 1% BSA) for 1 h, washed, and then a horseradish peroxidase (HRP)-conjugated phosphotyrosine (PY) antibody was added overnight. The antibody was removed, and the cells were washed again in TBS, exposed to an enhanced luminol ELISA substrate (Pierce Chemical EMD, Rockford, IL) and light emission was measured using a UV Products
(Upland, CA) BioChemi digital darkroom. Data were graphed as a percent of cells receiving growth factor alone, and IC$_{50}$ values were calculated from two to three separate experiments ($n = 8–24$) using non-linear regression dose-response relation analysis.

**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% CO$_2$ 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% CO$_2$ 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 IC$_{50}$ was defined as the compound concentration causing a 50% reduction in the increase in cell protein as compared with cultures without compound addition.

**Chorioallantoic membrane assay of angiogenesis**

The CAM assay is a standard assay for testing antiangiogenic agents. The CAM assay used in these studies was modified from a procedure by Sheu and Brooks and as published previously. Briefly, fertile leghorn chicken eggs (CBT Farms, Chestertown, MD) were
allowed to grow until 10 days of incubation. The proangiogenic factors human VEGF-165 and bFGF (100 ng each) were then added at saturation to a 6 mm microbial testing disk (BBL, Cockeysville, MD) and placed onto the CAM by breaking a small hole in the superior surface of the egg. Antiangiogenic compounds were added 8 h after the VEGF/bFGF at saturation to the same microbial testing disk and embryos allowed to incubate for an additional 40 h. After 48 h, the CAMs were perfused with 2% paraformaldehyde/3% glutaraldehyde containing 0.025% Triton X-100 for 20 sec, excised around the area of treatment, fixed again in 2% paraformaldehyde/3% glutaraldehyde for 30 min, placed on Petri dishes, and a digitized image taken using a dissecting microscope (Wild M400; Bannockburn, IL) at 7.5X and a SPOT enhanced digital imaging system (Diagnostic Instruments, Sterling Heights, MI). A grid was then added to the digital CAM images and the average number of vessels within 5–7 grids counted as a measure of vascularity. Sunitinib and semaxanib were used as a positive control for antiangiogenic activity. Data were graphed as a percent of CAMs receiving bFGF/VEGF only and IC_{50} values calculated from two to three separate experiments (n = 5–11) using non-linear regression dose-response relation analysis.
<table>
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<th>Compd.(Lab record book no)</th>
<th>EGFR Inhibition</th>
<th>CAM inhibition</th>
<th>VEGFR-2 inhibition</th>
<th>PDGFR-β inhibition</th>
<th>A431 cytotoxicity</th>
<th>MDA-MB-231</th>
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<td>123 (65)</td>
<td>335.3 ± 4.0</td>
<td>24.5 ± 4.0</td>
<td>34.2 ± 4.0</td>
<td>137.0</td>
<td>100.2</td>
<td>&gt;1000</td>
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<tr>
<td>124 (61)</td>
<td>146.2 ± 18.9</td>
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<td>15.4 ± 2.1</td>
<td>98.5</td>
<td>40.2 ± 8.2</td>
<td>193.2 ± 30.2</td>
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<tr>
<td>125 (70)</td>
<td>255.6 ± 30.2</td>
<td>30.1 ± 4.3</td>
<td>32.5 ± 5.0</td>
<td>193.1</td>
<td>47.2 ± 5.9</td>
<td>227.2 ± 4.1</td>
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<tr>
<td>156 (29)</td>
<td>100.3 ± 14.8</td>
<td>10.3 ± 1.9</td>
<td>7.9 ± 1.8</td>
<td>108.5</td>
<td>33.2 ± 5.8</td>
<td>227.2 ± 30.3</td>
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<tr>
<td>122 (46)</td>
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<td>20.6 ± 3.1</td>
<td>33.2 ± 4.1</td>
<td>183.2</td>
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<td>137 (66)</td>
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<td>30.1 ± 5.7</td>
<td>292.4 ± 39.1</td>
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<td>172 (79)</td>
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<td>7.8 ± 0.9</td>
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<tr>
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<td>&gt;200</td>
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**Table 3**: Inhibition of growth of β-III and P-gp overexpressing cells.

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<th>WT HeLa IC₅₀ (nM)</th>
<th>β-III overexpressing HeLa IC₅₀ (nM)</th>
<th>Parental OVCAR-8 IC₅₀ (nM)</th>
<th>P-gp overexpressing NCI/DR-RES IC₅₀ (nM)</th>
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<td>5.7 ± 0.4</td>
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<td>32.9 ± 5.8</td>
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**Tubulin immunofluorescence assay**

MDA-MB-435 cells were plated in chamber slides at ~30% confluency and allowed to attach overnight. Cells were then treated with drug at the IC$_{50}$ concentration for 2 h in serum free media. After 2 h, drug was removed, cells were washed and then fixed in 3.7% buffered formalin for 10 min. Fixed cells were then washed 3x with PBS, permeabilized with 0.5% Triton 100-X in PBS for 10 min at RT then blocked in 10% goat serum for 30 min at RT. Slides were washed again with PBS 3x and then incubated with anti-alpha-tubulin Alexa Fluor® 488 (Invitrogen, Carlsbad CA) in PBS containing 10% goat serum at a concentration of 2.5 μg/mL for 1 h at RT in the dark. After incubation, cells were washed, chambers removed, SlowFade Gold (Molecular Probes/Invitrogen, Carlsbad CA) added and a cover slip applied. Slides were imaged using a fluorescent microscope, Leica DM4000 B (Wetzlar, Germany) at EX488 nm/EM519 nm.

**Maximum tolerated dose in mice**

To determine the maximum tolerated dose (MTD) of compounds and drugs, a dose finding study was performed using BALBc/J mice (Jackson Laboratories, Bar Harbor, ME). Drugs were first dissolved at 50 mg/mL in DMSO and frozen in aliquots at -80 °C. Solutol-15 (BASF, Ludwigshafen, Germany) was melted at 60 °C for 5-10 min, then mixed in a ratio of 1 part DMSO/drug to 1.8 parts solutol-15 to 7.2 parts sterile dextrose 5% in water (D$_5$W). This solvent mixture was used for all drugs. Starting at 10 mg/kg and 15 mg/kg body weight (n = 2 mice per treatment), mice were weighed and doses increased in 10 mg/kg increments every other day until weight loss was observed. At this point the MTD was estimated to be
the approximate dose of first weight loss. The MTD of docetaxel was found to be 35 mg/kg, sunitinib 30 mg/kg and 126 [RM/AG-90·HCl] 50 mg/kg, temozolomide 30 mg/kg.

**U251 flank xenograft model**

Human U251 glioblastoma cells (500,000) in media were implanted into the lateral flank of 8 wk old male NCr athymic nu/nu nude mice (Charles River, Wilmington, DE). Tumor sizes (length, width, depth) were measured twice weekly. When volumes reached 75-100 mm³ (day 8 after implantation), treatment with drugs at their MTD (above) was begun and animal weights and tumor volumes measured twice weekly. At the end of the experiment, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors were removed, fixed in neutral buffered formalin, paraffin embedded, sectioned and sections stained against CD31/PECAM-1 using an antibody from Abcam (ab28364) and staining done using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA Vessel density was assessed by counting the number of CD31-positive vessels in a 200x microscope field in a blinded fashion and graphed as a percent vessel density of carrier treated animals.
Figure 1: Treatment with 126 [RM/AG-90·HCl] decreased primary tumor growth and tumor vascular density in U251 flank xenograft mouse model. * P<0.05, ** P<0.01, *** P<0.001 by one way ANOVA with Neuman-Keuls post test. U251 human glioma cells were implanted into the lateral flank of NCr athymic nu/nu nude mice at 500,000 cells, and the mice were treated with carrier temozolomide or 126 [RM/AG-90·HCl] twice weekly at their MTD’s until the end of the experiment. Data is representative of 6-8 animals. (A) Tumor size was determined as described in the legend of Figure 3. Statistics on this graph were two way ANOVA with repeated measures post test. (B) Graphical representation of percent change in animal weight as determined by measuring animal weight at the beginning and end of experiment.
Table 4. Cancer cell growth inhibitory activity (GI$_{50}$, nM) of 126 (RM/AG-90.HCl) (NCI 60 cell line panel)

<table>
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<th>Panel/ Cell line</th>
<th>GI$_{50}$ (nM)</th>
<th>Panel/ Cell line</th>
<th>GI$_{50}$ (nM)</th>
<th>Panel/ Cell line</th>
<th>GI$_{50}$ (nM)</th>
<th>Panel/ Cell line</th>
<th>GI$_{50}$ (nM)</th>
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<td>Leukemia</td>
<td></td>
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The biological evaluations of the analogs listed in the following tables 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.

**Cell Lines and Assays of Antitumor Drug Activities.** RFC- and FRR-null MTXRIIOuR2-4 (R2) CHO cells were gifts from Dr. Wayne Flintoff (University of Western Ontario) and were cultured in R-minimal essential medium (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), penicillin- streptomycin solution and L-glutamine at 37 °C with 5% CO₂. PC43-10 cells are R2 cells transfected with hRFC. RT16 cells are R2 cells transfected with human FRα, and D4 cells are R2 cells transfected with human FRβ. R2/hPCFT4 cells were prepared by transfection of R2 cells with an hPCFT cDNA, epitope tagged at the C-terminus with Myc-His6 (hPCFT-Myc-His6) and cloned in pCDNA3.1. All the R2 transfected cells (PC43-10, RT16, D4, R2/hPCFT4) were routinely cultured in R-MEM plus 1.5 mg/mL G418. Prior to the cytotoxicity assays (see below), RT16 and D4 cells were cultured in complete folate-free RPMI1640 (without added folate) for 3 days. KB human cervical cancer cells were purchased from the American Type Culture Collection (Manassas, VA), whereas IGROV1 ovarian carcinoma cells were a gift of Dr. Manohar Ratnam (Medical University of Ohio). Cells were routinely
cultured in folate-free RPMI1640 medium, supplemented with 10% fetal bovine serum, penicillin-streptomycin solution, and 2 mM L-glutamine at 37 °C with 5% CO₂. For growth inhibition assays, cells (CHO, KB, or IGROV1) were plated in 96 well dishes (~2500-5000 cells/well, total volume of 200 µL medium) with a broad range of antifolate concentrations. The medium was RPMI1640 (contains 2.3 µM folic acid) with 10% dialyzed serum and antibiotics for experiments with R2 and PC43-10 cells. For RT16, D4, KB, and IGROV1 cells, the cells were cultured in folate-free RPMI media with 10% dialyzed fetal bovine serum (Invitrogen) and antibiotics supplemented with 2 nM LCV. The requirement for FR mediated drug uptake in these assays was established in a parallel incubation including 200 nM folic acid. For R2/hPCFT4 cells, the medium was folate-free RPMI1640 (pH 7.2) containing 25 nM LCV, supplemented with 10% dialyzed fetal bovine serum (Invitrogen) and antibiotics. Cells were routinely incubated for up to 96 h, and metabolically active cells (a measure of cell viability) were assayed with Cell Titer-blue cell viability assay (Promega, Madison, WI), with fluorescence measured (590 nm emission, 560 nm excitation) using a fluorescence plate reader. Raw data were exported from Softmax Pro software to an Excel spreadsheet for analysis and determinations of IC₅₀s, corresponding to the drug concentrations that result in 50% loss of cell growth. For some of the in vitro growth inhibition studies, the inhibitory effects of the antifolate inhibitors on de novo thymidylate biosynthesis (i.e., TS) and de novo purine biosynthesis (GARFTase and AICARFTase) were tested by coincubations with thymidine (10 µM) and adenosine (60 µM), respectively. For de novo purine biosynthesis, additional protection experiments used AICA (320 µM) as a means of distinguishing inhibitory effects at
GARFTase from those at AICARFTase. For assays of colony formation in the presence of the antifolate drugs, KB cells were harvested and diluted, and 200 cells were plated into 60mm dishes in folate-free RPMI1640 medium supplemented with 2 nM LCV, 10% dialyzed fetal bovine serum, penicillin-streptomycin, and 2 mM L-glutamine in the presence of antifolate drugs. The dishes were incubated at 37 °C with 5% CO₂ for 10-14 days. At the end of the incubations, the dishes were rinsed with Dulbecco’s phosphate-buffered saline (DPBS), 5% trichloroacetic acid, and borate buffer (10 mM, pH 8.8), followed by 30 min incubation in 1% methylene blue in the borate buffer. The dishes were rinsed with the borate buffer, and colonies were counted for calculating percent colony-forming efficiency normalized to control.

**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 phosphate-buffered 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 [³H]folic 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 $[^3\text{H}]$folic acid binding by 50%. By definition, the relative affinity of folic acid is 1.

**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-morpholinepropanesulfonic acid (MES)-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 5 mM glucose) adjusted to pH 6.5, 6.0, or 5.5 for R2/hPCFT4 cells. In either case, uptakes of $[^3\text{H}]$MTX (0.5 $\mu$M) were measured over 2 min at 37 °C in the presence and absence of unlabeled antifolates (10 $\mu$M). Uptakes of $[^3\text{H}]$MTX 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 $[^3\text{H}]$MTX uptake in the presence and absence of the inhibitors. Kinetic constants ($K_t$, $V_{\text{max}}$) and $K_i$s were calculated from Lineweaver-Burke and Dixon plots, respectively.
**In Vitro GARFTase Enzyme Inhibition Assay.** Purified recombinant mouse GARFTase enzyme was a gift from Dr. Richard Moran (Virginia Commonwealth University, Richmond, VA). Briefly, enzyme activity was assayed spectrophotometrically at 37 °C using GARFTase (0.75 nM), α,β-GAR (11 μM), and coenzyme 10-formyl-5,8-dideazafolic acid (10 μM) in HEPES buffer (75 mM, pH 7.5) with or without antifolate inhibitor (10-30 000 nM). The absorbance of the reaction product, 5,8-dideazafolic acid, was monitored at 295 nM over the first minute as a measure of the initial rate of enzyme activity. IC\(_{50}\)s were calculated as the concentrations of inhibitors that resulted in a 50% decrease in the initial velocity of the GARFTase reaction.

**In Situ GARFT Enzyme Inhibition Assay.** Incorporation of [\(^{14}\)C]glycine into [\(^{14}\)C]FGAR, as an in situ measure of endogenous GARFTase activity, was described by Beardsley et al. and modified by Deng et al. For these experiments, KB cells were seeded in 4 mL of complete folate-free RPMI1640 plus 2 nM LCV in 60 mm dishes at a density of 2 × 106 cells per dish. On the next day, the medium was replaced with 2 mL of fresh complete folate-free RPMI1640 plus 2 nM LCV (without supplementing glutamine). Azaserine (4 μM final concentration) was added in the presence and absence of the antifolate inhibitors (0.1, 1, 10, 100, or 1000 nM). After 30 min, L-glutamine (final concentration, 2 mM) and [\(^{14}\)C]glycine (tracer amounts; final specific activity 0.1 mCi/L) were added. Incubations were at 37 °C for 15 h, at which time cells were washed (one-time) with ice-cold folate-free RPMI1640 plus serum. Cell pellets were dissolved in 2mL of 5% trichloroacetic acid at 0 °C. Cell debris was removed by centrifugation (the cell
protein contents in the pellets were measured), and the supernatants were extracted twice with 2 mL of ice-cold ether. The aqueous layer was passed through a 1 cm column of AG1 × 8 (chloride form), 100-200 mesh (Bio-Rad), washed with 10 mL of 0.5 N formic acid and then 10 mL of 4 N formic acid, and finally eluted with 8 mL of 1 N HCl. The elutants were collected and determined for radioactivity. The accumulation of radioactive FGAR was calculated as pmol per mg protein over a range of inhibitor concentrations. IC50s were calculated as the concentrations of inhibitors that resulted in a 50% decrease in FGAR synthesis.

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<th>RT16 (nM) FR</th>
<th>D4 (nM) FR</th>
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**Table 1.** IC<sub>50</sub> values (nM) for 5-substituted pyrrolo[2,3-d]pyrimidine antifolates and the clinically used classical antifolate PMX in RFC-, PCFT-, and FR-expressing Chinese hamster ovary (CHO) cell lines and KB human tumor cells. The data shown summarize results from 3 to 10 experiments. The results are presented as mean IC<sub>50</sub> values corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug.
Fig. 1 Protection studies for compounds 173 (RM/AG-293) and 174 (RM/AG-306)
The synthetic strategy required the substituted indole 30 which was synthesized in two steps from 3-bromo-2-chloronitrobenzene 28 by a potassium t-butoxide induced displacement with cyanoacetate to 29, followed by Zn-dust catalyzed reduction. Cyclocondensation of 30 with chloroformamidine in methylsulfone at 120 °C for 5 h or guanidine hydrochloride in DMF under reflux conditions did not afford 31.\(^7\)
Intermediate 3 was synthesized from commercially available 2,4-diamino-6-hydroxypyrimidine 2 and chloroacetaldehyde in sodium acetate-water solution. The cyclocondensation was regiospecific and afforded 3 in 82% yield. The 2-amino group was pivaloylated and then chloromercuration of 4 was carried out by adding mercuric acetate in glacial acetic acid, followed by the addition of saturated sodium chloride solution. The precipitated white solid was filtered and washed thoroughly with water to give a 10:1 mixture of 6-chloromercuri derivative 5 and 5-chloromercuri derivative in 50% overall yield. The resulting mixture of chloromercuri derivatives was treated with iodine in dichloromethane to afford the corresponding 6-iodo and 5-iodo derivatives from which the desired 6-iodo 6 was readily separated by column chromatography in 64% yield.
Compound 8 were readily prepared from 7 using the Vilsmeier reagent with DMF/POCl₃ in 70% yield. 5-Arylthiophene-2-carboxylate 9 were then synthesized by reacting β-chlorovinyl aldehyde 8 with mercaptoacetate in the presence of potassium carbonate. Then, two step, one pot palladium catalyzed borylation/Suzuki coupling reaction was attempted with iodo 6 utilizing bis-boronic acids but did not provide the desired compound 12.

The aldehyde 6 was alpha-brominated using bromine to yield 7 which was then cyclized using commercially available 2,4-diamino-6-hydroxypyrimidine to furnish 8 which was then pivylated to afford 9. Bromination of 9 was then attempted but did not give the desired product 10.
Commercially available acid 2 was esterified with ethanol to afford 3 which was then alkylated with 3-bromo-1-propanol to afford alkylated intermediate 4. The intermediate 4 were then oxidized using Dess-Martin Oxidation but gave a series of side products which could not be isolated.
Gangjee et al. reported\textsuperscript{12} the synthesis of the pivaloyl-protected pyrrolo[2,3-d]pyrimidine 2 before. A Mannich reaction was carried out on 2.\textsuperscript{13} The Mannich reagent 3 was prepared by the slow addition of 37% aqueous formaldehyde to a cold (0 °C) solution of N,N-dimethylamine in glacial acetic acid. Compound 4 was added to the cold reagent solution, and the mixture was heated to 50 °C for 48 h to afford 5 as a crude product in 55 % yield. Compound 5 was not purified and directly taken to next step which was hydrolysis using 1 N NaOH to afford 6. The peptide with (L)-diethyl glutamate esters using N-methylmorpholine and 6-chloro-2,4-dimethoxytriazine as the activating agents did not afford the final product 7.