Design and synthesis of pyrimido[4,5-b]indoles and furo[2,3-d]pyrimidines as single agents with combination chemotherapy potential or as inhibitors of tubulin or thymidylate synthase

Ravi Kumar Vyas Devambatla
DESIGN AND SYNTHESIS OF PYRIMIDO[4,5-b]INDOLES AND
FURO[2,3-d]PYRIMIDINES AS SINGLE AGENTS WITH COMBINATION
CHEMOTHERAPY POTENTIAL OR AS INHIBITORS OF TUBULIN OR
THYMIDYLATE SYNTHASE

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By
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ABSTRACT

DESIGN AND SYNTHESIS OF PYRIMIDO[4,5-b]INDOLES AND FURO[2,3-d]PYRIMIDINES AS SINGLE AGENTS WITH COMBINATION CHEMOTHERAPY POTENTIAL OR AS INHIBITORS OF TUBULIN OR THYMIDYLATE SYNTHASE

By
Ravi Kumar Vyas Devambatla

May 2015

Dissertation supervised by Professor Aleem Gangjee

This dissertation describes an introduction, background and research progress in the areas of multitargeted single agents and tubulin inhibitors in cancer chemotherapy and selective Toxoplasma gondii TS inhibitors for the treatment of toxoplasmosis.

Tubulin inhibitors are important antitumor agents that disrupt microtubule dynamics. Thymidylate synthase (TS) inhibitors prevent cell division by interfering with de novo thymidylate synthesis. Antiangiogenic agents target tumor angiogenesis crucial for tumor growth and metastasis. Under normal circumstances, angiogenesis is typically limited to tumor cells and is mediated by receptor tyrosine kinases (RTKs). Combination chemotherapies of RTK inhibitors with cytotoxic agents that target either TS or tubulin have shown significant promise and several preclinical and clinical studies with such combinations are in progress. Multitargeted single agents with dual antiangiogenic and
cytotoxic mechanisms could avoid the major limitations associated with cancer chemotherapy: multidrug resistance and dose limiting toxicities. This dissertation focuses on the design and synthesis of pyrimido[4,5-b]indoles and furo[2,3-d]pyrimidines as potential single agents with dual antiangiogenic and cytotoxic activities. These efforts led to the identification of structural features that are necessary for inhibition of RTKs and/or tubulin polymerization. Novel synthetic strategies were developed for efficient synthesis of 2,4-diamino-5-thioaryl-pyrimido[4,5-b]indoles and 4-anilino-5-methyl-furo[2,3-d]pyrimidines.

Taxanes and vinca alkaloids are widely used tubulin inhibitors in cancer chemotherapy. However, their clinical use is compromised by two major mechanisms of drug resistance: the overexpression of Pgp and βIII-tubulin. This dissertation describes the design and synthesis of pyrimido[4,5-b]indoles as tubulin inhibitors that circumvent Pgp and βIII-tubulin mediated resistance. This work identified the structural features crucial for tubulin inhibition for the pyrimido[4,5-b]indole scaffold.

Infection by Toxoplasma gondii can lead to toxoplasmosis in immune compromised patients such as organ transplant, cancer and AIDS patients. Current therapy involving combination of sulfadiazine and pyrimethamine is limited by drug resistance and treatment failures. The thymidylate synthase–dihydrofolate reductase enzyme is important for thymidylate synthesis in T. gondii, and hence can be targeted to treat T. gondii infection. TS is highly conserved across species and selectivity for tgTS over human TS is significantly more challenging. The present work provides an efficient synthesis of 2-diamino-4-oxo-5-thioaryl-pyrimido[4,5-b]indoles as selective tgTS inhibitors.
Dedicated to My Mom and Dad
ACKNOWLEDGEMENTS

I would never have been able to finish my dissertation without the guidance of my committee members, help from colleagues, and support from my parents and wife.

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<td>5,10-CH₂THF</td>
<td>$N^5,N^{10}$-Methylene tetrahydrofolate</td>
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>5-F-5'-dCR</td>
<td>5-Fluoro-5'-deoxycytidine</td>
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<tr>
<td>5-F-5'-dUR</td>
<td>5-Fluoro-5'-deoxyuridine</td>
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<tr>
<td>5-F-dUMP</td>
<td>5-Fluoro-2'-deoxyuridine 5'-monophosphate</td>
</tr>
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<td>5-F-dUTP</td>
<td>5-Fluoro-2'-deoxyuridine 5'-triphosphate</td>
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<tr>
<td>5-F-UTP</td>
<td>5-Fluorouridine triphosphate</td>
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<tr>
<td>AICARTFase</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleotide transformylase</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>CA1</td>
<td>Combretastatin A-1</td>
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<tr>
<td>CA1P</td>
<td>Combretastatin A-1 diphosphate</td>
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<tr>
<td>CA4</td>
<td>Combretastatin A-4</td>
</tr>
<tr>
<td>CA4P</td>
<td>Combretastatin A-4 phosphate</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>dCMP</td>
<td>2'-Deoxyctydine-5'-monophosphate</td>
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<td>DFG</td>
<td>Aspartate-Phenylalanine-Glycine</td>
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<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
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<tr>
<td>Acronym</td>
<td>Explanation</td>
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<td>DHFR</td>
<td>Dihydrofolate reductase</td>
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<td>DHPS</td>
<td>Dihydropteroate synthase</td>
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<td>DMAP</td>
<td>4-((N,N\text{-Dimethylamino})pyridine)</td>
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<td>DMF</td>
<td>Dimethylformamide</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dTMP</td>
<td>2'-Deoxythymidine-5'-monophosphate</td>
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<td>dTTP</td>
<td>2'-Deoxythymidine-5'-triphosphate</td>
</tr>
<tr>
<td>dUMP</td>
<td>2'-Deoxyuridine-5'-monophosphate</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FDA</td>
<td>U.S. Food and drug administration</td>
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<tr>
<td>FPGS</td>
<td>Folylpoly-(\gamma)-glutamate synthetase</td>
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<td>GARTFase</td>
<td>Glycinamide ribonucleotide transformylase</td>
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<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
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<tr>
<td>HER</td>
<td>human epidermal growth factor receptor</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>hTS</td>
<td>Human thymidylate synthase</td>
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<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MOE</td>
<td>Molecular Operating Environment</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolodinone</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>PDB</td>
<td>Protein data bank</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PLGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
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<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
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<tr>
<td>tgTS</td>
<td><em>T. gondii</em> thymidylate synthase</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin–layer chromatography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>TS–DHFR</td>
<td>Thymidylate synthase–dihydrofolate reductase</td>
</tr>
<tr>
<td>VDA</td>
<td>Vascular-disrupting agent</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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I. BIOLOGICAL REVIEW

A. Tubulin binding agents

A.1 Microtubules

Microtubules are long, filamentous protein polymers that form one of the major components of the cytoskeleton.\(^1\)

![Image of microtubule polymerization]

**Figure 1.** Polymerization of αβ-tubulin heterodimers (modified from ref. 2). αβ-Tubulin heterodimers first form a short microtubule nucleus followed by elongation and arrangement to form a microtubule.

Microtubules are composed of α- and β-tubulin heterodimers each with a molecular weight of about 50 kDa. The αβ-tubulin heterodimers polymerize “head-to-tail” between the α- and β-tubulin to form protofilaments, which are aligned in parallel to form hollow cylindrical microtubule structure (Figure 1).\(^2\) The resulting microtubule has an outer diameter of 24 nm, a wall thickness of 5 nm, and a length extendable to about 25 µm. Each microtubule has α- and β-tubulin on the plus (+) and minus (−) ends, respectively.
A.2 Microtubule dynamics

Microtubules exhibit two major forms of non-equilibrium dynamics, namely dynamic instability and treadmilling.

![Dynamic instability of microtubules](image)

**Figure 2.** Dynamic instability of microtubules (modified from ref. 2).

In dynamic instability, the individual microtubule ends switch between phases of lengthening and shortening (Figure 2). The growth and shortening of microtubules occur more rapidly and more extensively at the plus (+) end, where the β-tubulin is exposed, than at the minus (−) end, where α-tubulin is exposed. The microtubules undergo relatively long periods of slow lengthening, brief periods of rapid shortening and periods of attenuated dynamics or pause, when the microtubule neither grows nor shortens detectably. Dynamic instability is characterized by four main factors: rate of growth; rate of shrinkage; frequency of switching from the growth stage to shortening (known as ‘‘catastrophe frequency’’) and frequency of switching from shrinkage to growth (known as ‘‘rescue frequency’’).
The second dynamic behavior, known as microtubule treadmilling (Figure 3), involves net growth at the plus end of the microtubule and balanced net shortening at the minus end.\textsuperscript{4, 5} It results in an intrinsic flow of tubulin subunits from the plus end of the microtubule to the minus end and is particularly important in mitotic spindles.

Microtubule associated proteins (MAPs), proteins associated with microtubules, are important for the formation and stability of microtubules.\textsuperscript{6} The two nonequivalent ends of microtubules are structurally well organized. The slow growing minus end is generally anchored at the microtubule organizing center (MTOC) of the centrosome whereas the plus end is highly dynamic and switches between phases of growth and shrinkage.\textsuperscript{6}

The polymerization dynamics of microtubules involves binding and/or hydrolysis of guanosine 5'-triphosphate (GTP).\textsuperscript{3, 6} Each αβ-tubulin heterodimer has two GTP-binding sites: an exchangeable (E) site on β-tubulin and a non-exchangeable (N) site on α-tubulin that is always filled with GTP. Binding of GTP to the E-site of tubulin is required for microtubule polymerization whereas GTP hydrolysis is required for microtubule depolymerization.\textsuperscript{3}
Figure 4. Polymerization dynamics of microtubules (modified from ref. 2). During polymerization, tubulin-bound GTP disassociates as soon as the tubulin-bound GDP binds to the microtubule or shortly thereafter. Depolymerization occurs when the GTP cap is hydrolyzed.

GTP binding to β-tubulin causes straightening of the αβ-tubulin heterodimer to a conformation that promotes polymerization. The GTP-bound heterodimer attaches to the plus end of the microtubule and then the GTP is hydrolyzed to tubulin–GDP and inorganic phosphate (P$_i$) (Figure 4), providing energy for the addition of another tubulin heterodimer. A microtubule end containing tubulin bound GTP or GDP-P$_i$ is stable or “capped” against depolymerization. Hydrolysis of tubulin-bound GTP and the subsequent release of P$_i$ induce a conformational change in the tubulin that results in microtubule depolymerization. At the final stage of the polymerization process, the GTP cap dissociates and leaves a microtubule core of β-tubulin bound with GDP.

A.3 Role of microtubule dynamics in mitosis

During mitosis, the duplicated chromosomes of cells are divided into two identical sets prior to division into two daughter cells. The correct movements of the
chromosomes and their proper segregation to daughter cells require extremely rapid microtubule dynamics. Their role in mitosis and cell division makes microtubules an important target for anticancer drugs.\textsuperscript{1,9} 

**Figure 5.** Microtubule dynamics during the cell cycle (modified from ref. 10). Microtubules are shown in green.

Microtubule dynamics vary during the cell cycle, being least dynamic during the interphase and most dynamic during mitosis.\textsuperscript{10} Mitosis in most cells progresses rapidly and the highly dynamic microtubules in the spindle are required for all stages of mitosis (Figure 5). During the prophase of mitosis, microtubules become more dynamic and a
bipolar spindle-shaped array of microtubules is assembled outwards from the centrosome. In the prometaphase, the kinetochore microtubules attach to the poles and make vast growing and shortening excursions until they capture the chromosomes. In metaphase, chromosomes are aligned along the equator and have at least two microtubules extending from the kinetochores. When cells enter anaphase, the kinetochore microtubules shorten and pull chromosomes to the poles. During telephase and cytokinesis, the chromosomes arrive at the poles, the mitotic spindle disassembles and the parent cell divides into two daughter cells. As different stages of mitosis are dependent on microtubule dynamics (Figure 5), disruption of microtubule dynamics by microtubule targeting agents results in cell cycle arrest, eventually causing programmed cell death (apoptosis).

A.4 Classification of tubulin binding agents

Tubulin binding agents are structurally highly diverse and are mainly classified into two groups: microtubule-stabilizers and microtubule-destabilizers. Microtubule-stabilizers promote microtubule polymerization at high concentrations and include the taxanes and the epothilones. Microtubule-destabilizers induce microtubule depolymerization at high concentrations and include the vinca alkaloids and colchicine-site binding agents. However, at low antiproliferative concentrations, both these types of agents suppress microtubule dynamics leading to mitotic arrest and subsequent cell death. Tubulin binding agents are widely used for the treatment of both solid tumors and hematological malignancies.

Most microtubule targeting agents are divided into four classes based on their interactions within the taxane, vinca, colchicine, or the newly discovered maytansine site on tubulin.
A.4.1 Taxanes and epothilones:

Paclitaxel (Taxol®), docetaxel (Taxotere®), and the epothilones bind to the β-subunit in the interior of the microtubule, commonly referred to as the taxane site (Figure 6). The binding of paclitaxel leads to the stabilization of microtubules and an increase in net microtubule polymerization. Thus, the ability of the cells to break down the mitotic spindle during mitosis is inhibited by paclitaxel. With the spindle still in place, the parent cell cannot divide into daughter cells resulting in the inhibition of mitosis. Binding of paclitaxel stabilizes the microtubule by inducing a conformational change in β-tubulin which increases its affinity for adjacent tubulin molecules. Low concentrations of Taxol are sufficient to cause cell cycle arrest and induce apoptosis without changing microtubule mass. The IC₅₀ of paclitaxel for an antimitotic effect in HeLa cells is 8 nM while the IC₅₀ for an increase in microtubule polymerization is 80 nM. The taxoids are widely used in the treatment of breast, non-small cell lung, ovarian, head and neck and prostate cancers.

Figure 6. Taxane binding site and representative taxanes (modified from ref. 2).
Most antimitotic agents that induce microtubule polymerization, such as the epothilones, discodermolide and eleutherobin bind to the taxane binding site.\textsuperscript{11} Ixabepilone (Ixempra\textsuperscript{®}), which belongs to the epothilone class of taxane-site binders, was approved for the treatment of drug-refractory metastatic breast cancer in 2007.\textsuperscript{16}

**A.4.2 The vinca alkaloids:**

The vinca alkaloids, including vinblastine, vincristine, vindesine, and vinorelbine have a broad spectrum of activity and have been widely used in cancer chemotherapy for lymphomas, non-small-cell lung cancer and pediatric cancers.\textsuperscript{1}

![Diagram of Vinca Binding Site and Representative Vinca Alkaloids](image)

**Figure 7.** Vinca binding site and representative vinca alkaloids (modified from ref. 2).

The vinca alkaloids bind to the β-subunit on the exterior of the microtubule at a site commonly referred to as the vinca-binding domain (Figure 7). The rapid and reversible binding of vinblastine to soluble tubulin induces a conformational change that leads to tubulin self-association.\textsuperscript{12} Vinblastine binds with high affinity to the end of the microtubules but poorly to tubulin that is buried in the tubulin lattice. At high concentrations of 10–100 nM in HeLa cells, vinblastine depolymerizes microtubules and
destroys mitotic spindles. At low concentrations (~1 nM), vinblastine does not depolymerize microtubules, yet it powerfully blocks mitosis by suppression of microtubule dynamics. The binding of one or two molecules of vinblastine per microtubule is sufficient to reduce tubulin dynamics by about 50%, without causing appreciable microtubule depolymerization. Other antitubulin agents such as the halichondrins, cryptophycins, and dolastatins also bind at the vinca binding site. Eribulin mesylate (Halaven®) is a synthetic halichondrin derivative which was approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic breast cancer.

A.4.3 Colchicine-site agents:

![Colchicine binding site and combretastatins](image)

**Figure 8.** Colchicine binding site and combretastatins (modified from ref. 2).

The third class comprises a diverse collection of small molecules, including colchicine (Figure 8), combretastatins A-1 (CA1) and A-4 (CA4), 2-methoxyestradiol and podophyllotoxin. Colchicine is not used as an antitumor agent due to its toxicity at doses that produce antimitotic effects. However, colchicine (Colcrys®) is useful for the
treatment of gout and familial Mediterranean fever.\textsuperscript{22} Colchicine blocks or suppresses cell division by inhibiting the development of spindles when the nuclei are dividing.\textsuperscript{21} Therefore it inhibits the division of the nucleus and mitosis. Colchicine binds to soluble tubulin at a distinct site referred to as the colchicine binding site, which is on \(\beta\)-tubulin at the interface between \(\alpha\)- and \(\beta\)-tubulins (Figure 8).\textsuperscript{12, 13} Colchicine forms a poorly reversible tubulin–colchicine complex with tubulin, which copolymerizes into the microtubule ends along with free tubulin molecules. The ends continue to grow with the complex, but their dynamics are suppressed. These effects lead to cell cycle arrest and apoptotic cell death. Similar to the vinca alkaloids, colchicine site binders depolymerize microtubules at high concentrations but suppress microtubule dynamics at low concentrations. Although there are no clinically approved anticancer agents that bind to the colchicine site, several agents including 2-methoxyestradiol,\textsuperscript{23, 24} CA4 phosphate (CA4P, Fosbretabulin®)\textsuperscript{25-28} and CA1 diphosphate (CA1P, OXi4503®)\textsuperscript{29} have been evaluated in phase 1 and 2 clinical trials as anticancer agents either alone or in combination.

In addition to the antimitotic effect, combretastatins are potent vascular-disrupting agents (VDAs) that target endothelial cells and pericytes of the already established tumor vasculature.\textsuperscript{30, 31} CA4P rapidly depolymerizes the microtubules of endothelial cells, reduces blood flow to the tumor by 95% within one hour, increases vascular permeability and hemorrhage of peripheral vessels.\textsuperscript{32} Based on this property, both CA4 and CA4P have been evaluated as anti-vascular agents in phase 1 and 2 clinical trials.\textsuperscript{30, 33} Importantly, VDAs seem to damage tumor vasculature without significantly harming normal tissues. This selectivity of the VDAs for tumor vasculature can be attributed to
the differences between the mature vasculature of normal tissues and the immature or forming vasculature of tumors.\textsuperscript{32, 34} Also, the actin cytoskeleton in endothelial cells of immature tumor vasculature is underdeveloped, which is likely to make the cells more susceptible to damaging agents. In addition, the differences in rates of endothelial cell proliferation may also be a causative factor to the observed tumor selectivity.\textsuperscript{32}

\textbf{A.4.4 Maytansine-site agents:}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{maytansine.png}
\caption{Representative maytansine-site agents.}
\end{figure}

Recently, Prota \textit{et al.}\textsuperscript{14} demonstrated that maytansine (Figure 9) binds to a distinct site on β-tubulin designated as the “maytansine site”. Occupancy of this site inhibits tubulin polymerization by preventing the addition of new subunits at the plus ends of the microtubule, thus causing microtubule depolymerization. Phase 1 drug PM060184\textsuperscript{35} (Figure 9) and Rhizoxin F\textsuperscript{36} are the other microtubule depolymerizing agents that bind to the maytansine site. Maytansine is the cytotoxic component in the antibody drug conjugate Trastuzumab emtansine (Kadcyla\textsuperscript{®}), which was recently approved by the FDA for the treatment of HER2-positive advanced breast cancer.\textsuperscript{37}
A.5 Mechanisms of tumor resistance to tubulin binding agents

Multidrug resistance (MDR) severely limits the effectiveness of cancer chemotherapy, including tubulin binding agents. Clinically significant mechanisms of tumor resistance for these agents are drug efflux by ATP-binding cassette (ABC) transporters and expression expression of the βIII-isotype of tubulin.

ABC transporters such as P-glycoprotein (Pgp) are membrane-bound drug efflux proteins that reduce the cellular accumulation of substrates. Drug efflux by Pgp was identified as the chief mechanism of resistance to the vinca alkaloids and taxanes in vitro. For example, the potency of paclitaxel in Pgp overexpressing SKOV3 MDR-1-6/6 cell lines was 800-fold less than in parental SKOV3 cell lines. Overexpression of Pgp has also been reported in the clinical setting in several tumor types, particularly in patients who have received chemotherapy. Furthermore, Pgp expression is considered to be a prognostic indicator in certain cancers and is associated with poor response to chemotherapy. Attempts to reverse Pgp-mediated drug resistance by combining tubulin binding agents with Pgp inhibitors have been disappointing.

The other clinical mechanism of resistance to tubulin binding agents involves the expression of βIII-isotype of tubulin. βIII-Tubulin expression is strongly associated with clinical resistance to taxanes in multiple tumor types such as non-small cell lung, breast, ovarian and gastric cancers. Paclitaxel had 5-fold less activity in βIII-tubulin expressing HeLa cell lines compared to the parental cell line. Also, paclitaxel displayed less suppression of microtubule dynamics in βIII-tubulin expressing cells than in control cells. It was hypothesized that the resistance to taxanes was due to the absence of Ser275 on βIII-tubulin. Paclitaxel is considered to reach its binding site on β-tubulin by
diffusion through the pores in the microtubule wall. This process is stabilized by the formation of a hydrogen bond between 7-hydroxyl of paclitaxel and the $-\text{OH}$ of Ser275 of $\beta$-tubulin. In $\beta$III-tubulin, Ser275 is replaced by Ala275 thus possibly resulting in decreased binding of paclitaxel to microtubules in $\beta$III-tubulin expressing cell lines. Overexpression of $\beta$III-tubulin is also involved in clinical resistance to Vinca alkaloids in non-small cell lung\textsuperscript{45, 52} and ovarian\textsuperscript{53} cancers. Colchicine-site binding agents have been shown to circumvent $\beta$III-tubulin resistance,\textsuperscript{54, 55} which highlights the advantage of colchicine-site agents over taxanes and vinca alkaloids for cancer chemotherapy.

**B. Thymidylate synthase inhibitors**

**B.1 Thymidylate synthase**

Thymidylate synthase (TS), a key enzyme in folate metabolism, is present in almost all living organisms including humans, bacteria and protozoa.\textsuperscript{56} It catalyzes the reductive methylation of 2'-deoxyuridine 5'-monophosphate (dUMP) to 2'-deoxythymidine 5'-monophosphate (dTMP) (Figure 10), which is subsequently phosphorylated to 2'-deoxythymidine 5'-triphosphate (dTTP) by cellular kinases. The dTTP formed is utilized by deoxyribonucleic acid (DNA) polymerase and is incorporated into DNA. The TS catalyzed reaction is a key step in DNA biosynthesis and the only de novo biosynthetic pathway to dTMP.\textsuperscript{57} During TS catalysis, $N^5,N^{10}$-methylene tetrahydrofolate (5,10-CH\textsubscript{2}THF) acts as a one-carbon donor and is converted to dihydrofolate (DHF) (Figure 10).
Figure 10. De novo synthesis of dTMP by the enzyme TS. TS catalyzes the transfer of methylene group from 5,10-CH$_2$THF to dUMP resulting in the formation of dTMP and DHF.

B.1.1 Structure of TS

The TS enzyme (EC 2.1.1.45) is a homodimer consisting of two identical subunits each having a molecular weight of 30–35 kDa and a primary sequence of approximately 316 amino acids.\textsuperscript{56} It has two active sites, each formed by residues from monomers. The primary structures of several TS enzymes including those of humans, bacteriophages, and plants are known. TS enzymes are highly conserved in different species both in terms of structure and mechanism: 27 amino acids are completely conserved across all species and 165 (80\%) are conserved in more than 60\% of the organisms. In particular, the active (dUMP) site of TS involves 32 amino acid residues, of which 16 are conserved in all species.

In the protein data bank (PDB),\textsuperscript{58} several X-ray crystal structures of TS of various organisms are available in the native enzyme form or as complexes with substrates, products or inhibitors. Notably, the X-ray crystal structures of TS from several
prokaryotic species, including *Escherichia coli* (*E. coli*), and eukaryotes, such as *Homo sapiens*, *Lactobacillus casei* (*L. casei*, lc), and *Pneumocystis carinii* (*P. carinii*) are known in the literature.\textsuperscript{56} Also, X-ray crystal structures of human TS inhibitors such as pemetrexed and raltitrexed are known.\textsuperscript{59, 60} The existing crystal structures of TS in the native form or with inhibitors are important for the understanding of both the mechanism and the inhibition of TS.\textsuperscript{56} More importantly, they allow a structure-based rational design of TS inhibitors.

Through site-directed mutagenesis, the function of each residue in the substrate-binding site of TS has been studied. Cys198, Asn229, Arg178' and Arg179', Glu60 and Val316 (lcTS numbering) were determined to be the most important residues in dUMP binding site.\textsuperscript{56} During the TS catalysis, Cys198 is involved in the nucleophilic attack on C6 of the uracil ring resulting in the formation of a covalent bond between TS and dUMP, thus no mutation is tolerated at Cys198. Asn229 (lcTS numbering), another important residue in the active site, is involved in hydrogen bonding with the 4-oxo of dUMP.\textsuperscript{56} Thus substitution of Asn229 by other amino acids resulted in reduced or complete loss of catalytic activity. Asn229 also plays an important role in enzyme specificity. When Asn229 is replaced with Asp229, the enzyme is no longer a deoxyuridylate methylase, but a cytidylate methylase, which catalyzes the methyltransferase reaction with 2'-deoxycytidine 5'-monophosphate (dCMP) instead of dUMP.

Amino acid residues, Arg178' and Arg179' (lcTS numbering), belong to the opposite subunit and bind to the phosphate group of the deoxyribose ring.\textsuperscript{56} Replacement of these two amino acids reduced the catalytic activity. The importance of other amino acids in
the active site has also been studied. The C-terminal residue Val316 participates in the conformational change of the enzyme, which occurs after the covalent binding of cofactor 5,10-CH₂THF to the binary complex TS–dUMP. This conformational change is necessary for the catalytic reaction because it positions dUMP and the cofactor to facilitate the transfer of the methyl group. Pro196, Pro197 and His199 (lcTS numbering) are highly conserved amino acids but their substitution with other amino acids is well tolerated.

**B.1.2 Catalytic mechanism of TS**

The mechanism of catalysis by TS has been examined and is depicted in Figure 11. Sequential binding of substrate (dUMP) and cofactor 5,10-CH₂THF with TS enzyme induces a conformational change to form a non-covalent ternary complex (TS–dUMP–cofactor). In step A, Cys195 of human TS (hTS) attacks C6 of dUMP to form the enol. Protonation at the \( N^{10} \)-position of 5,10-CH₂THF results in the formation of the reactive \( N^5 \)-iminium species in step (B). The C5 position of the enol of dUMP reacts with the iminium ion of the activated cofactor to form an unstable covalent ternary complex in step (C).\(^6\) The proton at the C5-position of dUMP is abstracted by a base in the active site to provide enol in step (D). Abstraction of a proton from O4 of the enol results in the formation of an exocyclic methylene and also the release of the reduced cofactor in step (E). The enzymatic reaction is completed by the hydride transfer from C6 of the reduced cofactor to form dTMP. The modified cofactor, which serves as a methylene donor and reductant, is released from the active site of TS followed by release of the product, dTMP.
Figure 11. The catalytic mechanism of human TS (modified from ref. 61).
B.2 Inhibition of TS

Inhibition of TS depletes dTTP, one of the four building blocks required for DNA synthesis, thus leading to impaired DNA synthesis and repair.\textsuperscript{57} dTTP depletion also perturbs cellular levels of deoxynucleotides resulting in DNA damage.\textsuperscript{57, 62} Alternatively, TS inhibition causes accumulation of dUMP, and subsequent misincorporation of dUTP into DNA.\textsuperscript{57} These processes result in DNA double-strand breaks that lead to the initiation of apoptotic cell death, commonly referred to as “thymidine-less” cell death.\textsuperscript{57}

B.3 TS inhibitors in cancer chemotherapy

Inhibition of TS in tumor cells inhibits DNA biosynthesis resulting in thymidine-less cell death. Normal cells also require TS for DNA synthesis.\textsuperscript{56} Nonetheless, TS inhibitors are widely used in cancer chemotherapy and the reason for their tumor selectivity is attributed to differences in transport, metabolism and the rates of cell division for normal and some tumor cells.\textsuperscript{57} As tumor cells replicate much more rapidly than normal cells, they have a higher need for synthesis of deoxynucleotides than normal cells. Several TS inhibitors have been rationally designed to target the TS-binding site of either dUMP or cofactor 5,10-CH$_2$THF. TS inhibitors are classified broadly into two classes: dUMP-based TS inhibitors and antifolates.

B.3.1 dUMP-based TS inhibitors

dUMP-based TS inhibitors are antimetabolites that are analogs of dUMP, one of the substrates in TS catalysis.\textsuperscript{57} These inhibitors, upon bioactivation, either inhibit TS or are misincorporated into DNA and/or RNA resulting in the inhibition of DNA and/or RNA synthesis.
Figure 12. dUMP and representative dUMP-based TS inhibitors.

5-Fluorouracil (5-FU, Figure 12) is a dUMP-based inhibitor and represents the first class of clinically used TS inhibitors. The mechanisms of action of 5-FU include inhibition of TS, incorporation into DNA, and/or incorporation into RNA. 5-FU is a prodrug and must be bioactivated to various active nucleotides such as 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-F-dUMP, Figure 12), 5-fluoro-2'-deoxyuridine 5'-triphosphate (5-F-dUTP) and 5-fluorouridine triphosphate (5-F-UTP). 5-F-dUMP is a mechanism-based inactivator of TS since it forms a covalent complex with TS and the cofactor. Alternatively, 5-F-dUMP can be phosphorylated by a series of enzymatic reactions to 5-F-UTP, which is misincorporated into RNA and causes inhibition of RNA processing and function.

5-FU has been used for more than 50 years in the treatment of colorectal cancer. 5-FU remains one of the most widely used anticancer agents with a broad-spectrum activity against many solid tumors, including pancreas, breast, head and neck, gastric, and ovarian cancers. However, 5-FU causes toxicity to normal cells because the enzymes that activate it are not tumor selective. For instance, 5-FU causes diarrhea because of its activation in the intestines. Moreover, it is rapidly degraded by the dihydropyrimidine dehydrogenases in the liver, thereby limiting its oral bioavailability.
Capecitabine (Xeloda®) and tegafur (Figure 12) are prodrugs that are metabolized to 5-FU, which then gets converted to active nucleotides involved in DNA and RNA damage.\textsuperscript{57, 64} Rational drug design of capecitabine exploited the utilization of three tumor-overexpressing enzymes for its metabolism to to 5-FU. Capecitabine is readily absorbed in the gastro-intestinal tract and is converted to 5-fluoro-5′-deoxycytidine (5-F-5′-dCR) by carboxylesterases in the liver. 5-F-5′-dCR is then converted to 5-fluoro-5′-deoxyuridine (5-F-5′-dUR) by cytidine deaminase, which is highly expressed in both liver and tumor tissues. The final step involves the conversion of 5-F-5′-dUR by either thymidine phosphorylase or uridine phosphorylase to form 5-FU. Both thymidine phosphorylase and uridine phosphorylase are highly active in tumors than in normal tissues, thus resulting in tumor specificity to capecitabine. This Medicinal Chemistry approach resulted in capecitabine with improved oral bioavailability and tumor selectivity over 5-FU. Capecitabine is used clinically in the treatment of metastatic breast and colorectal cancers.\textsuperscript{65} It has also shown promising anticancer activity in patients with prostate, pancreatic, and ovarian cancers. Tegafur (Figure 12) is a prodrug that is bioactivated in the liver by cytochrome P-450 2A6 via 5′-hydroxylation to 5-FU.\textsuperscript{57}

B.3.2 Antifolates

Antifolates are classes of compounds that are structurally related to natural cofactor. TS-targeting antifolates are designed to attach to the binding site of the folate cofactor 5,10-CH\textsubscript{2}THF, involved in TS catalysis.\textsuperscript{57} They are competitive TS inhibitors, but unlike 5,10-CH\textsubscript{2}THF, are not bioactivated to form the iminium species. Antifolates are generally divided into two classes: Classical antifolates that contain a benzoyl L-glutamate side-chain and non-classical antifolates that lack the benzoyl L-glutamate.
\(N^{10}\)-Propargyl-5,8-dideazafolate (PDDF, Figure 13), was one of the first antifolates to be developed as a specific TS inhibitor.\(^6\) It was also the first quinazoline-containing TS inhibitor that entered phase I clinical trials. However, its development was abandoned due of its renal toxicity resulting from poor water-solubility.\(^6\) Replacement of the 2-amino group of PDDF with a 2-methyl group resulted in improved water solubility and decreased renal toxicity.

![Figure 13](image)

**Figure 13.** Cofactor 5,10-CH\(_2\)THF and representative antifolates as TS inhibitors.

Raltitrexed (Tomudex\(^\text{®}\), Figure 13) is the 2-methyl-containing classical antifolate derived from PDDF with decreased renal-toxicity but lower TS-inhibitory potency than PDDF.\(^6\)\(^7\) It is transported into cells via the reduced folate carrier (RFC) and undergoes rapid polyglutamylation by folylpoly-\(\gamma\)-glutamate synthetase (FPGS).\(^6\)\(^4\) Upon polyglutamylation, the potency of raltitrexed is increased by up to 100-fold and cellular retention is significantly prolonged.\(^6\)\(^4\)

In phase II and III trials, raltitrexed had good activity in patients with advanced colorectal and breast cancers.\(^6\)\(^8\) Unfortunately, raltitrexed caused life-threatening toxicity
attributed to gastrointestinal toxicity and myelosuppression. Raltitrexed was approved as first-line therapy for the treatment of advanced colorectal cancer in several European countries, Australia, Canada, and Japan.

Multiple mechanisms of resistance such as decreased uptake by RFC and/or decreased FPGS activity limit the clinical use of classical antifolates.\textsuperscript{61} Nonclassical lipophilic antifolates such as nolatrexed (Thymitaq\textsuperscript{®}) and plevitrexed (Figure 13) have been designed to circumvent these resistance mechanisms associated with classical antifolates. Plevitrexed contains a tetrazole moiety instead of the γ-carboxylic acid group and is transported into cells by RFC and passive diffusion but is not a substrate for FPGS.\textsuperscript{61} Nolatrexed is the first nonclassical TS inhibitor to reach phase II clinical trials.\textsuperscript{69} However, it showed minimal activity in patients with advanced hepatocellular carcinoma.

Pemetrexed ((Alimta\textsuperscript{®}), Figure 13) is a purported multitargeted antifolate that inhibits TS in addition to other folate-dependent enzymes including dihydrofolate reductase (DHFR) (very weakly), 5-aminomimidazole-4-carboxamide ribonucleotide transformylase (AICARTFase), and glycaminide ribonucleotide transformylase (GARTFase) (very weakly).\textsuperscript{70} It predominantly enters cells via the RFC under normal pHs and requires polyglutamylation for maximal inhibitory effects.\textsuperscript{64} Pemetrexed is approved in the USA for the treatment of advanced nonsquamous non-small cell lung cancer and malignant pleural mesothelioma. The main toxicities associated with pemetrexed are hematologic toxicity, gastrointestinal toxicity, fatigue, and skin rashes.\textsuperscript{71}

\textbf{B.4 Thymidylate synthase inhibition in toxoplasmosis}

Toxoplasmosis is a disease that affects the brain and the eyes and is caused by the intracellular parasite \textit{Toxoplasma gondii} (\textit{T. gondii}, tg).\textsuperscript{72} Humans generally acquire the
infection by ingestion of food or water that is contaminated with oocysts shed by cats or by eating raw or undercooked meat containing tissue cysts. According to the World Health Organization, the *T. gondii* parasite has infected up to a third of the world’s population. While toxoplasmosis is generally benign and goes unnoticed in immunocompetent individuals, it is a major opportunistic infection in immune compromised patients such as those with acquired immune deficiency syndrome (AIDS) and those receiving organ transplants or chemotherapy. *T. gondii* infection is one of the major causes of morbidity and mortality in HIV patients.

Protozoa, including *T. gondii*, contain a bifunctional TS–DHFR enzyme wherein the TS and DHFR exist on the same polypeptide. Recently, Sharma *et al.* solved the first three-dimensional structure of *T. gondii* TS–DHFR (tgTS–DHFR) with a resolution of 3.7 Å. The tgTS–DHFR enzyme is a homodimer in which each monomer has a molecular mass of 69 kDa. Each monomer consists of a TS domain (289 residues) on the C-terminal and a DHFR domain (252 residues) on the N-terminal. TS and DHFR domains are tethered together by a linker region (69 residues). The crystal structure and the overall sequence alignment also indicated that the active site of *T. gondii* TS (tgTS) is highly conserved. Cys489 of the TS domain is involved in the catalysis of dUMP to dTMP. The key residues important for the binding to PDDF (antifolate) are Ile402, Asp513, Leu516, Phe520, Arg603 and Met608. The tgTS–DHFR exhibits substrate channeling i.e. the dihydrofolate is directly transferred from the TS active site to the DHFR active site without entering bulk solution.

*T. gondii* relies on TS–DHFR for the synthesis of nucleotides essential for its survival. In addition, *T. gondii*, unlike humans, lacks salvage of thymidine thus tgTS
function is necessary for survival of the parasite.\textsuperscript{76} Since the TS–DHFR enzyme is crucial for \textit{T. gondii} survival, selective inhibition of tgTS represents a valid target to combat \textit{T. gondii} infection. However, achieving selective inhibition of tgTS over hTS is difficult because TS is highly conserved across species (much more than DHFR) and also there is significant homology between the active site residues of tgTS and hTS as there is across species.\textsuperscript{77, 78}

\textbf{C. Receptor tyrosine kinase inhibitors}

\textbf{C.1 Angiogenesis}

Angiogenesis is a complex physiological process in which new blood vessels are formed from existing vasculature.\textsuperscript{79} Under normal physiological conditions, angiogenesis is tightly regulated by a balance between proangiogenic and antiangiogenic factors and is only promoted during embryonic and post-embryonic development, reproductive cycle, and wound repair.\textsuperscript{80} However, upregulated angiogenesis has been described as one of the hallmarks of cancer, playing a crucial role in tumor growth, invasion, and metastasis. To grow beyond 2–3 mm\textsuperscript{3} in size, solid tumors require increased blood supply to fulfill their demand for nutrients, oxygen, and proteolytic enzymes.\textsuperscript{81} As a tumor grows in size, it also becomes increasingly hypoxic, and triggers the release of proangiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDGF) and epidermal growth factor (EGF). These growth factors act as proangiogenic signals to initiate angiogenesis.
Angiogenesis is initiated when resting endothelial cells are activated by a proangiogenic signal (Figure 14). Endothelial cells release membrane degrading enzymes leading to migration, proliferation and finally differentiation to form new microvessels. A scheme describing the angiogenic process is provided in Figure 14.

The new blood vessels grow and infiltrate into the tumor, thereby providing nutrients and oxygen to sustain tumor proliferation and a route for cancer cell dissemination. This results in tumor progression and metastases. The sustained growth of solid tumors is highly dependent on angiogenesis. Thus antiangiogenic agents have been widely used in the treatment of cancer. The present review will focus on receptor tyrosine kinases (RTKs) as potential targets for the inhibition of angiogenesis.

**Figure 14.** The angiogenic process (modified from ref. 81).
C.2 Receptor tyrosine kinases

Proangiogenic growth factors such as VEGF, PDGF and EGF bind to their corresponding growth factor receptors, commonly known as receptor tyrosine kinases (RTKs), and initiate signal transduction. The RTKs, namely vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor-β (PDGFR-β) and epidermal growth factor receptor (EGFR), are cell-surface receptors that transfer a phosphate group from adenosine 5'-triphosphate (ATP) to the hydroxyl group of tyrosine of specific proteins inside the cell. The RTKs possess an extracellular domain, a transmembrane domain and an intracellular kinase catalytic domain (Figure 15).

![Diagram of RTK signaling pathways](image)

**Figure 15.** Signaling pathways of RTKs (modified from ref. 86).
Binding of the growth factor to the extracellular domain of RTKs results in receptor dimerization followed by autophosphorylation of the tyrosine residues within the cytoplasmic domain. Autophosphorylation then triggers a cascade of downstream cell signaling pathways (Figure 15) mediated by kinases such as Ras kinase, phosphoinositide 3-kinase (PI3K), mitogen/extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK). Under normal physiological conditions, these signaling pathways are tightly monitored and regulated. Disregulation of these signaling pathways has been linked to malignancy and significantly contributes to the hallmarks of cancer: insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, tumor invasion and metastases. Thus, inhibition of these RTKs offers a key therapeutic strategy for cancer therapy.

C.3 Inhibitors of receptor tyrosine kinases

![Figure 16. Representative RTK inhibitors in the clinic.](image)

Several antiangiogenic agents such as sunitinib (Sutent®), sorafenib (Nexavar®) and erlotinib (Tarceva®) are currently approved for use in cancer patients (Figure 16).
Imatinib mesylate (Gleevec®, Figure 2) was the first RTK inhibitor approved in the U.S. for chronic myeloid leukemia. A number of RTKs have been recognized to be involved in tumor-induced angiogenesis. The key RTKs in angiogenesis include the VEGFR, PDGFR and EGFR families.

**VEGFR.** Three types of VEGFRs have been identified, VEGFR-1, VEGFR-2 and VEGFR-3. Among these, VEGFR-2 has been recognized as the principal receptor that mediates VEGF stimulation in angiogenesis. The VEGF family consists of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). VEGF is an important growth factor that is involved in angiogenesis and vasculogenesis (i.e., the formation of the embryonic circulatory system). VEGFRs are almost exclusively expressed on endothelial cells. In addition, VEGFRs are overexpressed in several tumor types with different expression patterns, ligand specificity and cellular/physiological effects of these receptors. Targeted inhibition or disruption of VEGFR-2 produces an abrogation of angiogenesis, decreased endothelial cell survival and decreased tumor growth. Several inhibitors of VEGFR-2 including sunitinib (Figure 16) and semaxanib have displayed antiangiogenic activity. Sunitinib (Sutent®) is a multi-RTK inhibitor (VEGFRs, PDGFRs and c-kit) important in the treatment of renal, pancreatic, and gastrointestinal cancers. Sorafenib (Nexavar®) is also a multi-RTK inhibitor (VEGFR-2, VEGFR-3, PDGFR-β and Raf kinase) used in the treatment of renal, hepatocellular and thyroid cancers.

**PDGFR.** PDGFR family is comprised of two receptors, PDGFR-α and PDGFR-β, which are activated upon binding to one of the growth factors PDGF-AA, -BB, -CC, -DD, and -AB. PDGFRs are expressed on pericytes, smooth muscle cells that provide
mechanical support to the vasculature. PDGFR plays an important role in angiogenesis by stimulating the formation of microvascular pericytes, thereby stabilizing the newly formed blood vessels. They are also involved in the induction of VEGF secretion and consequently, angiogenesis. Sunitinib and sorafenib are the representative PDGFR-β inhibitors in the clinic.

**EGFR.** The EGFR family of receptors contains four members: EGFR-1 (later referred to as EGFR; also identified as Erb-B1, or human EGF receptor HER-1), HER-2 (Erb-B2), HER-3 (Erb-B3), and HER-4 (Erb-B4). Among these, HER-2 lacks a known endogeneous ligand and HER-3 lacks kinase activity. The ligand for EGFR, EGF, controls a pathway that is linked to cell proliferation, migration, and differentiation. Overexpression of EGFR, its growth factors, and aberrant EGFR tyrosine kinase activity lead to increased tumor cell proliferation, survival and invasiveness. Inhibition of EGFR signaling has been shown to promote selective apoptosis in tumor endothelial cells. As a result, EGFR has been extensively studied and targeted by small molecule inhibitors and monoclonal antibodies. Erlotinib (Tarceva®) is an EGFR inhibitor used to treat pancreatic and non-small cell lung cancers.

**C.4 ATP-binding site of RTKs**

Several X-ray crystal structures of RTKs are available in the PDB database. All kinases share a catalytic domain that contains the ATP-binding site. The ATP-binding site of RTKs has been utilized as a promising target for rational drug design. Structural homology and diversity among the ATP-binding sites of kinases has allowed the building of pharmacophore models for rational drug design. The overall three-dimensional structure of the kinase domain is conserved throughout the protein kinase family.
N-terminal lobe consists of a twisted β-sheet of five antiparallel β-strands and one α-helix. The C-terminal lobe is made up of four β-strands and eight α-helices. Availability of ATP-bound or inhibitor-bound RTK crystal structures has enabled a detailed analysis of the catalytic site, its binding pockets in both active and inactive states as well as the modes of binding of RTK inhibitors.¹⁰⁷

**RTK catalytic cleft.** The catalytic cleft of the RTKs usually consists of two regions commonly referred to as the front cleft and the back cleft.¹¹⁰ While the ATP-binding site occupies the front cleft of the catalytic domain, the back cleft comprises of the elements responsible for the regulation of phosphorylation of peptide substrates. These two regions share a border that includes the aspartate-phenylalanine-glycine (DFG) motif and the β3 segment. A gatekeeper residue and a β3-lysine residue form a gate between the front and back cleft. Access to the back cleft is controlled by kinase gatekeeper residues. Small amino acid residues such as threonine and alanine or bulky gatekeeper residues such as phenylalanine, leucine or methionine control entry to binding in the back pocket and selectivity for inhibitors. The β3-lysine which is conserved in all kinase enzymes can adopt varied conformations in different protein kinases and helps in anchoring the α- and β-phosphates of ATP in the active state.

In a fully active state, protein kinases adopt a DFG-in conformation where the side chain of the DFG aspartate is directed into the ATP-binding site and the aromatic ring of the phenylalanine is positioned in the back cleft.¹¹⁰ The aspartate in DFG motif chelates with Mg²⁺ and also helps in orienting the γ-phosphate of ATP for its transfer in the active DFG-in conformation.¹⁰⁷,¹¹⁰ The aromatic side chain of phenylalanine in the active DFG-
in motif is in contact with αC. This contact in many active kinases facilitates the formation of a Lys-Glu ion pair for kinase catalysis.\textsuperscript{107}

In the inactive state, the DFG motif is in either a DFG-in or DFG-out conformation.\textsuperscript{110} In the DFG-out conformation, the phenylalanine aromatic ring is positioned in the ATP pocket and the aspartate residue of the DFG motif is in the back cleft.\textsuperscript{107}

\textbf{The front cleft.} The front cleft includes the ATP-binding site and small, non-ATP contact regions. The ATP site is broadly divided into the following subregions depending on the binding mode (Figure 17).\textsuperscript{107}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure17.png}
\caption{ATP-binding site of RTKs (modified from ref. 111). ATP is shown in magenta.}
\end{figure}

1. Adenine region: Adenine region is predominantly hydrophobic and is involved in binding various inhibitor scaffolds.\textsuperscript{112} This region 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. At least one of these key hydrogen bonds are utilized by several RTK inhibitors in binding to the RTKs. Other backbone amide groups in the Hinge region can serve as hydrogen bond donors or acceptors for inhibitor binding.

2. Ribose-binding region: The ribose pocket accommodates the sugar moiety of ATP and is adjacent to a hydrophilic, solvent exposed region. This pocket includes three hydrophobic residues (Ile, Val and Leu) which have been traditionally exploited in the design of EGFR inhibitors.\textsuperscript{111}

3. Phosphate-binding region: This region is covered by Asp, Lys and Asn residues and the DFG motif. This region is highly flexible, hydrophilic and solvent exposed, and is therefore considered less important in improving inhibitor affinity and potency.\textsuperscript{112}

4. Hydrophobic region II: This pocket is not used by ATP and serves as an entrance for ligand binding. The residues and conformation of this pocket vary depending on the kinase. Thus, this region has been used to obtain selectivity in the design of kinase inhibitors.\textsuperscript{112}

**The back cleft.** In addition to the ATP-binding site, the back cleft provides important binding regions. ATP does not bind in the pockets in the back cleft.\textsuperscript{110, 111}

1. Hydrophobic region I: The hydrophobic pocket in the back cleft adjacent to the adenine pocket is called Hydrophobic region I or Back pocket I (BP-I). This pocket has been explored in the design of inhibitors to gain selectivity for kinase targets with small gatekeeper residues.\textsuperscript{110}

2. In addition to BP-I, additional binding pockets BP-II, BP-III, and BP-IV can also be accessed by inhibitors depending on the binding state of the RTKs.\textsuperscript{107}
Major progress has been made in the rational drug design of RTK inhibitors targeting the ATP-binding site of RTKs. The front catalytic cleft of all kinase enzymes is accessible to ligand binding. Small molecule inhibitors that target the front cleft use a core scaffold to recognize the hinge region. The core scaffold is then substituted to extend into the different pockets of the ATP-binding site resulting in improved binding affinity and selectivity for RTKs.\textsuperscript{113}

D. Single agents in combination chemotherapy

Multitargeted single agents or designed multiple ligands are defined as rationally designed single chemical entities that can selectively target two or more biological targets or processes.\textsuperscript{114} The most commonly used strategy in the design of multitargeted agents is the hybrid drug design which involves combining structural elements from different compounds that bind to their respective targets. If the pharmacophores for the respective targets overlap, the common structural features of the lead compounds can be "merged" resulting in a multitargeted single agent.

Targeting different pathways in tumor cells using multitargeted agents can increase therapeutic effectiveness, as this strategy may for example prevent cancer cells from developing resistance.\textsuperscript{115} Other advantages include a lower risk of drug–drug interactions \textit{in vivo} and improved patient compliance due to less medications required.\textsuperscript{116} A challenging factor in the design of multitargeted single agents is optimization of 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 the targets).\textsuperscript{114}
Several multitargeted drug strategies have been studied in cancer chemotherapy.\textsuperscript{117,118} One of the clinical strategies that have been successful is the combination of antiangiogenic and cytotoxic agents.\textsuperscript{119-124} Multitargeted single agents that combine antiangiogenic effects by RTK inhibition and cytotoxic effects, are reviewed in the following section.

**D.1 Dual RTK and TS inhibition**

![Figure 18. Single agents with dual RTK and TS inhibitory activity.](image)

2,4-Diamino-5-thioaryl-pyrimido[4,5-b]indoles 1 and 2 (Figure 18) have been reported by Gangjee and coworkers\textsuperscript{125} as single agents with antiangiogenic and cytotoxic activities. These compounds inhibit VEGFR-2 and PDGFR-β for antiangiogenic effects and TS for cytotoxic effects with inhibitory potencies comparable or better than the standard compounds: semaxanib (VEGFR-2 inhibitor), DMBI (PDGFR-β inhibitor) and raltitrexed (hTS inhibitor). In COLO-205 metastatic colon cancer xenograft mouse models, compound 1 significantly decreased tumor growth, liver metastases and angiogenesis better than DMBI, remarkably without any toxicity.
D.2 Dual RTK and tubulin inhibition

Figure 19. Single agents with dual RTK and tubulin inhibitory activity.

Gangjee and coworkers\textsuperscript{55, 126} used hybrid drug design of RTK and tubulin inhibitors that led to the discovery of 3 and 4 (Figure 19) as single agents with antiangiogenic and cytotoxic activities. Both 3 and 4 had cytotoxic activity attributed to the inhibition of tubulin. While compound 3 inhibited both VEGFR-2 and PDGFR-\(\beta\) kinases for antiangiogenic effects, compound 4 had inhibition against VEGFR-2. Compounds 3 and 4 significantly reduced tumor size and vascularity in xenograft and allograft murine models and were superior to docetaxel and sunitinib, without any toxicity.

D.3 Dual RTK and DHFR inhibition

Figure 20. Single agents with dual RTK and DHFR inhibitory activity.
Gangjee et al.\textsuperscript{127} reported 5-substituted, 2,4-diamino-furo[2,3-\textit{d}]pyrimidines 5 and 6 (Figure 20) as inhibitors of multiple RTKs and DHFR with antiangiogenic and antitumor activities. A 2:1 \textit{E/Z} mixture of 5 and the \textit{E}-isomer of 6 demonstrated dual VEGFR-2 and PDGFR-\(\beta\) inhibitory activity comparable to semaxanib and AG1295 as standard compounds, respectively. In a B16 melanoma mouse model, compounds 5 and 6 were as active as the standard antitumor agent methotrexate, both against primary tumors and metastases without any major toxicities.
II. CHEMICAL REVIEW

This section will review the chemistry relevant to the work described in this dissertation. It provides synthetic approaches to the following heterocyclic ring systems.

A. Pyrimido[4,5-b]indoles
B. Furo[2,3-d]pyrimidines

A. Pyrimido[4,5-b]indoles

Methods employed in the synthesis of pyrimido[4,5-b]indoles can be broadly divided into three classes:

1. Synthesis of pyrimido[4,5-b]indoles from indole precursors
2. Synthesis of pyrimido[4,5-b]indoles from pyrimidine precursors
3. Miscellaneous methods

A.1 Synthesis of pyrimido[4,5-b]indoles from indole precursors

![Figure 21](image.png)

**Figure 21.** Disconnection strategy for pyrimido[4,5-b]indoles from indole precursors.

Different methods used in the synthesis of pyrimido[4,5-b]indoles from indoles or tetrahydroindoles utilize a disconnection strategy as shown in Figure 21.

Treatment of 1,2-dichloro-3-nitrobenzene 7 with ethyl cyanoacetate provided 8,125 which underwent reductive cyclization to the indole 9 (Scheme 1). Cyclocondensation of
9 with chlorformamidine hydrochloride afforded 2-amino-4-oxo-5-chloro-pyrimido[4,5-b]indole 10.

**Scheme 1.** Synthesis of 2-amino-pyrimido[4,5-b]indoles from indoles

Alternatively, 2-amino-4-oxo-pyrimido[4,5-b]indoles can be synthesized by a two-step procedure as shown in Scheme 2 below.

**Scheme 2.** Sequential two-step synthesis of 2-amino-pyrimido[4,5-b]indoles
Reaction of the indole 11 (Scheme 2) with cyanamide and conc. HCl provided amidine intermediate 12, which cyclized under alkaline conditions to 2-amino-4-oxo-6,7-dimethoxy-pyrimido[4,5-b]indole 13.\textsuperscript{128}

**Scheme 3.** Synthesis of the 2-unsubstituted pyrimido[4,5-b]indole 17.

![Scheme 3](image)

Analogous to 4-chloroindole derivative 9 in Scheme 1, the 5-chloroindole 16 (Scheme 3) was obtained from 1,3-dichloro-4-nitrobenzene 14.\textsuperscript{129} Compound 16 was cyclized using formamide to afford 2-unsubstituted 4-oxo-6-chloro-pyrimido[4,5-b]indole 17.

**Scheme 4.** Versatile synthesis of 2-substituted pyrimido[4,5-b]indoles
Showalter et al.\textsuperscript{130} reported a versatile procedure for the preparation of 2-substituted 4-oxo-pyrimido[4,5-\(b\)]indoles 19, 22 and 23 from ethyl 2-amino-1\(H\)-indole-3-carboxylate 18 (Scheme 4). Compound 18 was cyclized using formamide under basic conditions to 2-unsubstituted pyrimido[4,5-\(b\)]indole 19. Treatment of 18 with cyanamide and acetonitrile under acidic conditions provided intermediates 20 and 21, which were cyclized using 1N NaOH to 2-amino- and 2-methyl-pyrimido[4,5-\(b\)]indoles 22 and 23, respectively.

**Scheme 5.** Venugopalan strategy\textsuperscript{128} for the synthesis of 2-substituted pyrimido[4,5-\(b\)]indoles

Venugopalan et al.\textsuperscript{128} reported the synthesis of 2-substituted pyrimido[4,5-\(b\)]indoles from ethyl 2-amino-4,5-dimethoxy-indole carboxylate 11 (Scheme 5). Treatment of 11 with ethyl carboxyisothiocyanate gave 24, which cyclized to the 2-thio-4-oxo-pyrimido[4,5-\(b\)]indole 25 under basic conditions. Methylation of 25 afforded the 2-
methylthio-pyrimido[4,5-\textit{b}]indole 26, which upon reduction gave the 2-unsubstituted pyrimidoindole 27. For the synthesis of 2-unsubstituted pyrimido[4,5-\textit{b}]indoles, this strategy presents an alternative to the approach described in Schemes 3 and 4, in which 2-unsubstituted pyrimidoindoles 17 (Scheme 3) and 19 (Scheme 4) were synthesized by a direct reaction of indoles with formamide.

**Scheme 6.** Synthesis of pyrimido[4,5-\textit{b}]indoles via Pd-catalyzed amidation and cyclization of 2-haloindoles

Kumar \textit{et al}.\textsuperscript{131} synthesized pyrimido[4,5-\textit{b}]indoles from 2-haloindoles via palladium-catalyzed amidation and cyclization. Coupling of the 3-formyl- or 3-acetyl-2-haloindoles 28 and amides 29 provided 30 in 80–94\% yield (Scheme 6). Treatment of 30 with ammonium formate resulted in the formation of pyrimido[4,5-\textit{b}]indoles 31. According to the proposed mechanism for 30\textsuperscript{\rightarrow}31, ammonium formate thermally decomposes and releases ammonia which attacks 3-carbonyl of 30 and generates the
“imine” intermediate, which then cyclizes to provide the desired pyrimido[4,5-b]indole 31.

In Schemes 1–6 above, indoles were utilized to obtain pyrimido[4,5-b]indoles. Schemes 7 and 8 below present synthesis of pyrimido[4,5-b]indoles from tetrahydroindoles.

**Scheme 7.** Synthesis of pyrimido[4,5-b]indoles from tetrahydroindoles

The tetrahydro-1H-indole 33 (Scheme 7) was obtained by the treatment of 2-hydroxycyclohexanone 32 with benzylamine and malononitrile.\(^{132}\) Compound 33 was cyclized using formic acid to the 4-oxo-tetrahydropyrimido[4,5-b]indole 34. Chlorination of the 4-oxo of 34 followed by displacement with 3-chloroaniline afforded 35. Oxidation of 35 using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and subsequent removal of the benzyl group provided pyrimido[4,5-b]indole 36.

Müller *et al.*\(^ {133}\) synthesized a series of chiral pyrimido[4,5-b]indole derivatives 41 and 42 (Scheme 8). Reaction of chiral tetrahydroindoles 37 with benzonitrile and sodium
methoxide yielded the 2-phenyl-4-amino-tetrahydropyrimido[4,5-b]indoles 38. On the other hand, treatment of 37 with benzoil chloride provided 40, which was subsequently cyclized to the 2-phenyl-4-oxo-tetrahydropyrimido[4,5-b]indoles 41. Dehydrogenation of the tetrahydroindoles 38 and 41 furnished the desired pyrimido[4,5-b]indoles 39 and 42, respectively.

**Scheme 8.** Synthesis of 2-phenyl-pyrimido[4,5-b]indoles from tetrahydroindoles

\[
\begin{align*}
\text{NC} & \, \xrightarrow{\text{PhCN, NaOMe}} \, \text{PhCN} \\
\text{H}_2\text{N} & \, \xrightarrow{i/\text{PrOH}} \, \text{NH}_2 \\
\text{R} & \, \xrightarrow{\text{77\% Cl}} \, \text{Cl} \\
\text{O} & \, \xrightarrow{\text{P}_2\text{O}_5} \, \text{N} \\
\text{Ph} & \, \xrightarrow{\text{10\% Pd/C}} \, \text{Ph} \\
\text{N} & \, \xrightarrow{\text{1-methyl-naphthalene, reflux, 70\%}} \, \text{N} \\
\end{align*}
\]

**A.2 Synthesis of pyrimido[4,5-b]indoles from pyrimidine precursors**

**Figure 22.** Disconnection strategies for pyrimido[4,5-b]indoles from pyrimidine precursors.
Different methods used in the synthesis of pyrimido[4,5-b]indoles from pyrimidines employ disconnection strategies A or B as shown in Figure 22.

**Scheme 9.** Pyrimido[4,5-b]indol-2,4-diones from Fischer indole cyclization of 6-(arylhydrazino)uracils

A series of pyrimido[4,5-b]indoles using a facile Fischer-type cyclization were synthesized by G. E. Wright (Scheme 9). Treatment of 6-aminouracil 43 with phenyl hydrazines 44 provided 6-(arylhydrazino)uracils 45–47. Compounds 45 and 46 underwent acid–mediated cyclization to 48 and 49 respectively, whereas 47 gave an equal mixture of regioisomers 50 and 51. The scope of this reaction was extended to the \( N^1 \)- and/or \( N^3 \)-methyl 6-(arylhydrazino)uracils 52–54 or the \( N \)-methyl aniline derivative 55, resulting in the synthesis of pyrimido[4,5-b]indol-2,4-diones 56–59, respectively.

The cyclization reaction is analogous to a classical Fischer indole synthesis. The mechanism probably involves \( N^6 \)-protonation followed by nucleophilic attack by the ortho phenyl carbon on the uracil 5-carbon and cleavage of the \( N-N \) bond.
Rearomatization of the phenyl moiety results in the formation of a 5-membered ring by a favored 5-exo-trig cyclization. Finally, the expulsion of the uracil amino group gives rise to the pyrimido[4,5-b]indole. In the formation of 59 from 55, the N-methyl on the aniline moiety is retained in the product, which indicates that the uracil 6-amino group is expelled as ammonia in the final step of the cyclization.

**Scheme 10.** Application of Nenitzescu reaction in the synthesis of pyrimido[4,5-b]indoles

The Nenitzescu reaction was utilized by Dotzauer et al.\textsuperscript{135} to prepare pyrimido[4,5-b]indoles via a one-step procedure from 1,4-benzoquinone 60 and 6-aminopyrimidines 61 (Scheme 10). Compounds 61 were treated with excess of 1,4-benzoquinone 60 in boiling glacial acetic acid to provide 6-hydroxy-pyrimido[4,5-b]indoles 62 via a mechanism similar to the Nenitzescu reaction. According to the proposed mechanism, the enone of
60 reacts with 6-aminopyrimidine 61 and forms a Michael adduct 62. Intermediate 62 is oxidized to 63 by benzoquinone 60, which in turn is reduced to hydroquinone 64. Intramolecular cyclization of 63 and final hydrogenation by hydroquinone 64 results in the formation of 2,4-disubstituted 6-hydroxy-pyrimido[4,5-b]indoles 65.

Scheme 11. Synthesis of pyrimido[4,5-b]indole-2,4-diones from 4-azidouracils

Lapachev et al.\textsuperscript{136} reported the conversion of 4-azidouracils to pyrimido[4,5-b]indoles (Scheme 11). The synthesis commenced by reacting \( N,N \)-disubstituted ureas 66 and 67 with phenyl malonic acid 68 in the presence of acetic anhydride to obtain 1,3-disubstituted barbituric acids 69 and 70, respectively. The 4-oxo of 69 and 70 were regiospecifically chlorinated to afford 71 and 72, which were then converted to 4-azidouracils 73 and 74, respectively. Thermolysis of the azidouracils 73 and 74 in boiling xylene resulted in the formation of the pyrimido[4,5-b]indole-2,4-diones 75 and 76, respectively. Hydrogenolysis of the \( N \)-benzyl of 76 afforded the 1,3-unsubstituted pyrimido[4,5-b]indole-2,4-dione 77.
Schemes 12 and 13 below employ disconnection strategy B (Figure 22) for the synthesis of pyrimido[4,5-b]indoles via the intramolecular cyclization of arylamino- or cyclohexy lamino-substituted pyrimidines.

**Scheme 12.** Large-scale synthesis of pyrimido[4,5-b]indole 85 from trichloropyrimidine

Mauragis *et al.*\(^{137}\) developed a reliable large-scale process to produce >100 kg of the pyrimido[4,5-b]indole 85 (Scheme 12). The synthesis commenced with displacement of the 2- and 4-chloro of 2,4,6-trichloropyrimidine 78 with pyrrolidine 79 to obtain 80. Displacement of the remaining chloro group in 80 with the lithium amide of 81 provided 82. Conversion of 82 to the tetrahydropyrimido[4,5-b]indole 84 was accomplished by alkylation with bromocyclohexanone 83 and subsequent cyclization. Dehydrogenation of 84 using 5% Pd/C afforded the pyrimido[4,5-b]indole 85.

*Zhang* *et al.*\(^{138}\) synthesized various pyrimido[4,5-b]indoles via Pd-catalyzed cyclization of 4-anilino-5-iodopyrimidines 88 (Scheme 13). Compounds 88 were
prepared from 4-chloro-5-iodopyrimidine 86 and appropriate anilines 87. Palladium-catalyzed cyclization of the 5-iodo of 88 provided pyrimido[4,5-b]indoles 89 in moderate to good yields.

**Scheme 13.** Synthesis of pyrimido[4,5-b]indoles via palladium-catalyzed cyclization

![Scheme 13](image_url)

R' = H or Me  
R = 6-Et, 6-OMe, 6-CO₂Me, 6-SO₂NMe₂, 6-Br, 7-Br, 5,6,7-triOMe

### A.3 Miscellaneous methods

Schemes 14–17 discuss the synthesis of pyrimido[4,5-b]indoles from oxindoles. Adib et al.¹³⁹ reported microwave-assisted synthesis of 2,4-diaryl-pyrimido[4,5-b]indoles from oxindoles (Scheme 14).

**Scheme 14.** Microwave-assisted synthesis of pyrimido[4,5-b]indoles from oxindoles

![Scheme 14](image_url)

Oxindoles 90 (X = H or Br) react with a variety of aryl nitriles of general structure 91 under microwave irradiation and solvent-free conditions to produce the corresponding pyrimido[4,5-b]indoles 92 in good to high yields (Scheme 14).¹³⁹
Scheme 15. Synthesis of 2-substituted pyrimido[4,5-b]indoles from oxindole

Borovik et al. developed a versatile procedure for the synthesis of 2-substituted 4-phenyl-pyrimido[4,5-b]indoles 97a–d from oxindole 93 (Scheme 15). Condensation of oxindole 93 with benzaldehyde afforded 94, which was treated with triethyloxonium tetrafluoroborate to obtain the 2-ethoxy(3-benzylidene)indolenine tetrafluoroborate 95. Compound 95 was cyclized with substituted amidines 96 to provide 2-substituted 4-phenyl-pyrimido[4,5-b]indoles 97a–d.

Scheme 16 provides the utilization of 2-amino-4-phenyl-pyrimido[4,5-b]indole 97a in the synthesis of various 2-substituted pyrimido[4,5-b]indoles 98 and 100a–c.\textsuperscript{140} Diazotization of 2-amino-pyrimidoindole 97a furnished 2-oxo-pyrimido[4,5-b]indole 98, which was chlorinated to obtain 99. Displacement of the 2-chloro of 99 with appropriate nucleophiles afforded 2-substituted pyrimido[4,5-b]indoles 100a–c.

**Scheme 17.** One-pot multi-component synthesis of pyrimido[4,5-b]indoles

\[
\begin{align*}
\text{R}^1 &= \text{H, Me, COMe} \\
\text{X} &= \text{O, S} \\
\text{R}^2 &= \text{H, 4-Me, 4-OME, 4-Cl, 4-Br, 4-NO}_2, 2\text{-OH}
\end{align*}
\]

2-Oxo- or 2-thio-substituted 4-aryl-pyrimido[4,5-b]indoles 104 (Scheme 17) were synthesized from a one-pot multi-component reaction of oxindoles 101, urea or thiourea 102 and aryl aldehyde 103 under solvent-free conditions (Scheme 17)\textsuperscript{141}. The synthesis employed ytterbium triflate as the catalyst.

Molina et al.\textsuperscript{142} employed an aza-Wittig type reaction in the synthesis of pyrimido[4,5-b]indole 110 (Scheme 18). Diazide 107 was prepared by condensation of o-azidobenzaldehyde 105 with ethyl azidoacetate 106. Staudinger reaction between the diazide 107 and triphenyl phosphine gave bis(iminophosphorane) 108. Treatment of 108 with p-tolyl isocyanate in toluene at reflux provided pyrimido[4,5-b]indole 110.

The conversion of 108 to 110 (Scheme 18) can be rationalized in terms of an initial aza-Wittig reaction between the iminophosphorane group linked to the aromatic ring and the isocyanate to give a highly reactive carbodiimide 109a. Intermediate 109a undergoes
cyclization by nucleophilic attack by the β-carbon (on the ester side chain) leading to the zwitterionic intermediate 109b, which undergoes further transformations to indole 109c. An aza-Wittig reaction of 109c with a second equivalent of aryl isocyanate gives the carbodiimide 109d, which subsequently cyclizes to pyrimido[4,5-b]indole 110 upon nucleophilic attack of the 2-amino group on the central carbon atom of the carbodiimide moiety.

**Scheme 18.** Synthesis of pyrimido[4,5-b]indole 110 using an aza-Wittig type reaction

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

Synthetic strategies that are used in the synthesis of furo[2,3-\textit{d}]pyrimidines can be broadly classified into two strategies:

1. Strategy I: From furan precursors
2. Strategy II: From pyrimidine precursors
B.1. Furo[2,3-d]pyrimidines from furan precursors

Figure 23. Disconnection strategies for furo[2,3-d]pyrimidines from furan precursors.

Different methods used in the synthesis of furo[2,3-d]pyrimidines from furan precursors utilize disconnections A or B (Figure 23). For disconnection A, the precursors to cyclization are generally 2-amino-furan-3-nitriles. Disconnection B involves 2-aminofurans and 1,3,5-triazines as precursors.

Scheme 19. Synthesis of 4-amino-furo[2,3-d]pyrimidines from 2-amino-furan-3-nitriles

Karl Gewald\textsuperscript{143} reported the first synthesis of furo[2,3-d]pyrimidines starting from a furan precursor. \(\alpha\)-Hydroxy ketones \textsuperscript{111} and \textsuperscript{112} were treated with malononitrile to afford 2-amino-3-cyanofurans \textsuperscript{113} and \textsuperscript{114} respectively (Scheme 19). Condensation of \textsuperscript{113} and \textsuperscript{114} with formamide provided 4-amino-furo[2,3-d]pyrimidines \textsuperscript{115} and \textsuperscript{116} respectively.

The aforementioned strategy to obtain furo[2,3-d]pyrimidines from 2-amino-furan-3-nitriles has been utilized by several research groups. Miyazaki and coworkers\textsuperscript{144} used this approach to synthesize 4-amino-5-aryl-furo[2,3-d]pyrimidines \textsuperscript{120} (Scheme 20).
**Scheme 20.** Synthesis of 4-amino-5-aryl-furo[2,3-\(d\)]pyrimidines

Treatment of 1-aryl-2-bromoethanone 117 (Scheme 20) with potassium formate and aqueous sodium bicarbonate provided 118.\(^{144}\) Compound 118 was treated with malononitrile to afford 2-amino-5-aryl-furan-3-nitriles 119, which were then heated to reflux with formamide to obtain 120 in 69–74% yield.

**Scheme 21.** Miyazaki strategy\(^{145}\) for the cyclization of 2-amino-3-cyanofurans to furo[2,3-\(d\)]pyrimidines

Miyazaki et al.\(^{145}\) reported an alternate synthesis of 2-unsubstituted 4-amino-5-aryl-furo[2,3-\(d\)]pyrimidines 122 (Scheme 21) by treating the furan 121 with triethyl orthoformate, followed by amination and base-catalyzed cyclization.
Scheme 22. Dimroth rearrangement in the synthesis of $N^4$-substituted 5,6-diaryl-furo[2,3-d]pyrimidines

Han and coworkers$^{146}$ reported a novel synthesis of $N^4$-substituted 5,6-diaryl-furo[2,3-d]pyrimidines 129–132 by microwave irradiation of the formamidine derivative 123 and substituted amines (Scheme 22). Compound 123 was reacted with DMF and benzene sulfonyl chloride to obtain the formamidine derivative 124. Compound 124 reacts with amines 125–127 or benzo[d][1,3]dioxol-5-amine 128 and undergoes Dimroth rearrangement to provide 129–132, respectively.

Schemes 19–22 focused on synthesis of furo[2,3-d]pyrimidines with no substitution at the 2-position. Scheme 23 below presents the synthesis of 2-methyl-furo[2,3-d]pyrimidines by Dave et al.$^{147}$ Acid-mediated condensation of the furan 114 with acetonitrile afforded 2-methyl-4-amino-5,6-diphenyl-furo[2,3-d]pyrimidine 133.
Scheme 23. Synthesis of 2-methyl-4-amino-furo[2,3-\textit{d}]pyrimidine 133

![Synthesis of 2-methyl-4-amino-furo[2,3-\textit{d}]pyrimidine 133](image)

Schemes 19–23 provided strategies for the synthesis of 4-amino-furo[2,3-\textit{d}]pyrimidines. Schemes 24 and 25 below focus on strategies toward the synthesis of 4-oxo-furo[2,3-\textit{d}]pyrimidines.

Scheme 24. Synthesis of 4-oxo-furo[2,3-\textit{d}]pyrimidines

![Synthesis of 4-oxo-furo[2,3-\textit{d}]pyrimidines](image)

Foloppe and coworkers\textsuperscript{148} performed condensation of the furan 114 (Scheme 24) and in situ generated acetic formic anhydride to obtain the \textit{N}-formyl derivative 134, which underwent thermal cyclization to 4-oxo-5,6-diphenyl-furo[2,3-\textit{d}]pyrimidine 135.

\(\alpha\)-Chloro-\(\beta\)-keto esters 136 and 137 (Scheme 25) reacted with ethyl cyanoacetate under basic conditions to form ketonitriles, which were readily cyclized to tetrasubstituted furans 138 and 139, respectively.\textsuperscript{149} Treatment of 138 and 139 with formamide under acidic conditions afforded furo[2,3-\textit{d}]pyrimidines 140 and 141,
respectively. Hydrolysis to acids 142 and 143 followed by decarboxylation gave 4-oxo-6-aryl-furo[2,3-\textit{d}]pyrimidines 144 and 145, respectively. Compounds 144 and 145 represent examples of 5-unsubstituted 6-aryl-furo[2,3-\textit{d}]pyrimidines from furan precursors.

**Scheme 25. Synthesis of 6-aryl-furo[2,3-\textit{d}]pyrimidines 144 and 145**

![Scheme 25 diagram]

Dang and Liu\textsuperscript{150} utilized disconnection strategy B (Figure 23) for the synthesis of furo[2,3-\textit{d}]pyrimidines from 2-aminofurans (Scheme 26).

**Scheme 26. Synthesis of furo[2,3-\textit{d}]pyrimidine 148 using Diels–Alder reaction**

![Scheme 26 diagram]
Diels–Alder reaction of 2-aminofuran 146 and 1,3,5-triazine 147 provided furo[2,3-\(d\)]pyrimidine 148 (Scheme 26). The transformation proceeds through a series of reactions involving Diels–Alder reaction to give intermediate 149, followed by the elimination of ammonia to afford intermediate 150, which then undergoes a retro Diels–Alder reaction to 148.

### B.2. Furo[2,3-\(d\)]pyrimidines from pyrimidine precursors

![Figure 24](image)

**Figure 24.** Disconnection strategy for furo[2,3-\(d\)]pyrimidines from pyrimidine precursors.

Synthesis of furo[2,3-\(d\)]pyrimidines from pyrimidine precursors utilize 2-hydroxypyrimidines as intermediates (Figure 24). Schemes 27–32 represent examples that utilize 4-hydroxypyrimidines as precursors for the synthesis of furo[2,3-\(d\)]pyrimidines.

**Scheme 27.** Synthesis of 2,6-dimethyl-furo[2,3-\(d\)]pyrimidine 154

Condensation of diethyl propargyl malonate 151 (Scheme 27) and acetamidine hydrochloride 152 under basic conditions gave 2,4-dihydroxy pyrimidine 153,\(^{55}\) which
underwent acid-catalyzed intramolecular cyclization to 2,6-dimethyl-4-oxo-furo[2,3-\(d\)]pyrimidine 154 in 83% yield.

**Scheme 28. Synthesis of 4,6-dimethyl-furo[2,3-\(d\)]pyrimidines**

Condensation of β-ketoester 155 (Scheme 28) with amidines 156 afforded 5-acetonyl pyrimidines 157.\(^{151}\) Similar to Scheme 27, compound 157 was cyclized under acidic conditions to provide 4,6-dimethyl-2-substituted furo[2,3-\(d\)]pyrimidines 158.

Unlike Schemes 27 and 28 that use acid-catalyzed cyclization, Sakamoto and coworkers\(^{152}\) employed basic conditions for the intramolecular cyclization to obtain the furo[2,3-\(d\)]pyrimidine 161 (Scheme 29) below.

**Scheme 29. Synthesis of furo[2,3-\(d\)]pyrimidine 161 from 5-iodopyrimidinol**

Sonogashira coupling of the 5-iodopyrimidinol 159 and phenylacetylene afforded intermediate 160 (Scheme 29),\(^{152}\) which was converted to 2,4-dimethyl-6-phenyl-furo[2,3-\(d\)]pyrimidine 161 using triethylamine. Sonagashira coupling is also a key step in construction of the furan moiety in Schemes 30 and 31 below.
Scheme 30. Versatile synthesis of tetra-substituted furo[2,3-\(d\)]pyrimidines from 5-iodopyrimidinols

\[
\begin{align*}
\text{NH} & \quad \text{O} \quad \text{OEt} \\
\begin{array}{c}
\text{162} \\
R^1 = \text{Me, Ph}
\end{array} & \quad \text{NaOEt} & \quad \text{EtOH} & \quad 85-98\% \\
\begin{array}{c}
\text{164} \\
R^1
\end{array} & \quad \text{I}_2, \text{NaOH} & \quad \text{49-90\%} \\
\begin{array}{c}
\text{165}
\end{array}
\end{align*}
\]

Liu et al.\textsuperscript{153} employed metal-catalyzed intramolecular cyclization in the synthesis of tetra-substituted furo[2,3-\(d\)]pyrimidines 169 (Scheme 30). The 2-substituted 5-iodopyrimidinols 165 were obtained by the condensation of amidines 162 with \(\beta\)-ketoester 163 to 164 and subsequent iodination. Sonogashira coupling of iodopyrimidinols 165 and acetylenes 166 provided 167, which upon Pd-catalyzed intramolecular cyclization with aryl iodides 168 gave 169.

Scheme 30 above utilizes two metal-catalyzed coupling reactions to obtain furo[2,3-\(d\)]pyrimidines from 5-iodopyrimidinols. Petricci and coworkers\textsuperscript{154} employed microwave irradiation to synthesize furo[2,3-\(d\)]pyrimidines from 5-iodopyrimidinols in a single step (Scheme 31). Sonogashira coupling of 2,6-disubstituted 5-iodopyrimidinols 170 and propargyl alcohol under microwave conditions provided 2,4,6-trisubstituted furo[2,3-\(d\)]pyrimidines 172 via intermediate 171.\textsuperscript{154}
Scheme 31. Microwave-assisted Sonagashira coupling in the synthesis of furo[2,3-d]pyrimidines

![Diagram](attachment:image1.png)

Scheme 32. Synthesis of 2-oxo-furo[2,3-d]pyrimidine from uracil

![Diagram](attachment:image2.png)

Eger et al.\textsuperscript{155} reported a novel synthesis of 2-oxo-furo[2,3-d]pyrimidine 177 (Scheme 32) via intramolecular cyclocondensation of 5-(2-bromovinyl)-uracil 176. Uracil 173 was converted to hydroxymethyluracil and subsequently oxidized to 5-formyluracil 174. Compound 174 was reacted with malonic acid to afford 5-(2-carboxyvinyl)uracil 175, which was decarboxybrominated to 176. Cyclization of 176 to the 2-oxo-furo[2,3-d]pyrimidine 177 was achieved using potassium tert-butoxide.
III. STATEMENT OF THE PROBLEM

The present work deals with the following three broad areas:

A. Combination chemotherapy potential in single agents

B. Inhibition of tubulin

C. Selective inhibition of *T. gondii* thymidylate synthase

A. Combination chemotherapy potential in single agents

Angiogenesis – the process of formation of new blood vessels from existing vasculature – is essential for tumor growth and metastasis. When a tumor grows beyond 2 mm$^3$, it requires nutrients and oxygen for its growth and survival and thus initiates angiogenesis. Under hypoxic conditions, tumors secrete proangiogenic growth factors such as VEGF, PDGF, and EGF, key mediators of angiogenesis. These growth factors bind to their respective RTKs (VEGFR, PDGFR-β and EGFR) and stimulate the process of angiogenesis resulting in tumor growth, survival and metastases. Agents that circumvent angiogenesis by inhibition of RTKs have established a new paradigm in cancer chemotherapy. RTK inhibitors that function by inhibition of a single RTK are prone to resistance by numerous mechanisms including redundant pathways, point mutations in the ATP-binding site and upregulation of additional RTKs. Consequently, multi-RTK inhibition in cancer chemotherapy has emerged as a promising approach and its validity has been highlighted by the approval of several multi-RTK inhibitors including sorafenib (inhibits VEGFR-2, VEGFR-3, PDGFR-β and Raf kinase) and sunitinib (inhibits VEGFRs, PDGFRs and c-kit). However, the antiangiogenic treatment only prevents blood supply to the tumor but does not destroy cancer cells. Thus
antiangiogenic agents need to be combined with radiotherapy or chemotherapy to form an effective therapy and achieve synergistic effects.\textsuperscript{159}

Inhibitors of TS and tubulin are widely used cytotoxic agents in cancer chemotherapy. While inhibition of the enzyme TS prevents \textit{de novo} synthesis of nucleotides crucial for DNA synthesis,\textsuperscript{57} tubulin inhibitors target microtubule dynamics involved in mitosis.\textsuperscript{1}

TS catalyzes the \textit{de novo} synthesis of dTMP from dUMP utilizing 5,10-CH\textsubscript{2}THF as the cofactor.\textsuperscript{56} Because of its vital role in DNA synthesis and cell growth, TS is a viable target for several clinically used cancer chemotherapeutic agents.\textsuperscript{57} Nucleoside–based inhibitors such as 5-FU and capecitabine have found extensive utility in ovarian, breast, colon, and several other cancers alone and in combinations and are a mainstay in cancer chemotherapy.\textsuperscript{63, 65} Antifolates, including raltitrexed and pemetrexed, are TS inhibitors clinically used, alone or in combination, in the treatment of mesotheliomas, non-small cell lung and advanced colorectal cancers.\textsuperscript{68, 70}

Since microtubule dynamics plays a crucial role in mitosis and cell division,\textsuperscript{1} microtubule targeting agents represent an important class of anticancer agents. Tubulin binding agents such as paclitaxel and Vinca alkaloids are widely used to treat solid tumors and hematological malignancies.\textsuperscript{1, 8, 9}

Combination chemotherapy with antiangiogenic agents and cytotoxic agents is more effective in cancer treatment than either agent alone.\textsuperscript{159, 160} Single agents with dual antiangiogenic and cytotoxic activities significantly decreased tumor growth, tumor metastasis and angiogenesis superior to docetaxel and sunitinib in xenograft mice models, remarkably without any toxicity.\textsuperscript{125, 126} Such single agents with multiple mechanisms of
action are commonly referred to as designed multiple ligands and have several pharmacokinetic and pharmacodynamic advantages. These agents could potentially avoid drug–drug interactions and pharmacokinetic problems associated with two or more agents. In addition, they could prevent or delay the emergence of resistance and not cause overlapping toxicities. Most significantly, single agents with dual cytotoxic and antiangiogenic activities simultaneously target rapidly proliferating tumor cells and the tumor vasculature. Also, such single agents could afford synergic effects as they can exert their cytotoxic effect as soon as or even during transient tumor vasculature normalization caused by the antiangiogenic component. As a result, structural design of such multitargeted single agents should allow the cytotoxicity to be manifested as soon as the antiangiogenic effects are operable. Thus the cytotoxic component of these agents need not be as potent as conventional chemotherapeutic agents. Dosing of such an antiangiogenic agent with comparatively lower cytotoxic activity would mimic metronomic chemotherapy, which utilizes more frequent and low-dose administrations of cytotoxic agents compared with conventional chemotherapy. Several clinical trials attest to the safety and efficacy of using antiangiogenic agents such as sunitinib and sorafenib with metronomic doses of cytotoxic agents. The single agents offer other advantages such as decreased cost and increased patient compliance, which can play a major part in the clinical success of a therapy.

The antiangiogenic component of single agents targets RTK-overexpressing endothelial cells and as a result, is typically targeted to tumor cells under normal circumstances. In contrast, the cytotoxic component interferes with tumor cell division with less selectivity over rapidly dividing normal cells present in bone marrow, hair and
cells lining the mouth and gut.\textsuperscript{168} Therefore, a key challenge in the design of single agents with a cytotoxic component is that the cytotoxic component should only destroy tumor cells that are compromised via the antiangiogenic effect but should not cause toxicity to normal cells not affected by the antiangiogenic effect.\textsuperscript{125} As a result, the cytotoxic component of these single agents need not be as potent as conventional chemotherapeutic agents and hence should avoid dose-limiting toxicities\textsuperscript{168} associated with cytotoxic agents.

Single agents with dual antiangiogenic and cytotoxic activities significantly decreased tumor growth, tumor metastasis and angiogenesis in xenograft mice models, remarkably without any toxicity.\textsuperscript{55, 125-127} The antiangiogenic effects of these compounds were due to inhibition of RTKs and the cytotoxic effects were due to inhibition of thymidylate synthase\textsuperscript{125} or tubulin\textsuperscript{55, 126} or dihydrofolate reductase\textsuperscript{127}. In these studies, VEGFR-2, PDGFR-\(\beta\) and EGFR were chosen as the targets for antiangiogenic effects because of their crucial role in angiogenesis. Additionally, the successful clinical and preclinical combinations of TS inhibitors (for e.g., capecitabine, pemetrexed) or tubulin inhibitors like paclitaxel with antiangiogenic agents was also an important factor in selecting TS and tubulin as the potential cytotoxic targets.\textsuperscript{121, 169-172}

**A.1 Design of 2,4-diamino-5-arylthio-9H-pyrimido[4,5-b]indoles as single agents with dual TS and RTK inhibitory potential**

Gangjee \textit{et al.}\textsuperscript{125} reported pyrimido[4,5-b]indoles \textbf{1} and \textbf{2} (Figure 25) as dual inhibitors of thymidylate synthase and multiple RTKs. In COLO-205 mice xenografts, compounds \textbf{1} and \textbf{2} significantly inhibited tumor growth, tumor vasculature and liver metastasis better than docetaxel and remarkably with no toxicity.\textsuperscript{125} Compounds
178–180\textsuperscript{173} (Figure 25) are analogs of 1 with variations at 5-position and were previously synthesized.

![Chemical structures](image)

**Figure 25.** Lead compounds 1, 2 and 178–180 as dual TS and RTK inhibitors.

**Table 1.** Inhibition of TS, RTKs and angiogenesis by 1, 2 and 178–180

<table>
<thead>
<tr>
<th>Compd</th>
<th>Inhibition of hTS (µM)</th>
<th>Inhibition of EGFR (nM)</th>
<th>Inhibition of VEGFR-2 (nM)</th>
<th>Inhibition of PDGFR-β (nM)</th>
<th>Inhibition of CAM angiogenesis (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.54</td>
<td>15.1 ± 2.5</td>
<td>22.6 ± 2.7</td>
<td>2.8 ± 0.3</td>
<td>28.2 ± 2.9</td>
</tr>
<tr>
<td>2</td>
<td>0.39</td>
<td>10.41 ± 1.9</td>
<td>56.3 ± 8.2</td>
<td>40.3 ± 6.7</td>
<td>20.3 ± 5.2</td>
</tr>
<tr>
<td>178</td>
<td>0.48</td>
<td>8.3 ± 1.9</td>
<td>29.9 ± 4.8</td>
<td>&gt;200</td>
<td>18.3 ± 3.2</td>
</tr>
<tr>
<td>179</td>
<td>n.d.\textsuperscript{a}</td>
<td>16.2 ± 3.2</td>
<td>46.7 ± 8.1</td>
<td>&gt;200</td>
<td>1.2 ± 0.04</td>
</tr>
<tr>
<td>180</td>
<td>0.14</td>
<td>20.8 ± 3.1</td>
<td>182.2 ± 34.2</td>
<td>60.5 ± 7.2</td>
<td>29.0 ± 3.0</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
<td>1.3 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
<td></td>
<td>29.1 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}n.d. not determined

Table 1 shows inhibitory data for 1, 2 and 178–180 against human TS and kinases EGFR, VEGFR-2 and PDGFR-β. Kinase inhibition was performed in cell-based assays and therefore, it should be noted that cellular permeability might play a role in the activities of these compounds.
Figure 26. Series I.

Compounds 1a–7a (Series I, Figure 26) were designed in this study to improve the RTK–inhibitory activity while retaining low submicromolar TS inhibition. Compounds 1a and 2a with 4'-F and 4'-CF$_3$ groups, respectively, were designed to evaluate the contribution of the para-electron-withdrawing moiety on biological activity. Compounds 3a and 4a were designed to determine the importance of the methyl group at the 4'-position for 2. In chorioallantoic membrane (CAM) assay, compound 179 with 3',4'-dichloro group had antiangiogenic activity comparable to sunitinib and was 15-fold more active in this assay than the 4'-chloro analog 178. This increase in activity might be due to either electronics and/or lipophilicity of the additional 3'-chloro group. Novel 3',4'-dimethyl analog 5a with lipophilicity similar to 179 was designed to evaluate the role of electronics and lipophilicity at 3'-position. The 4'-OMe analog 180 had lower RTK inhibitory activity than the 4'-methyl compound 2. To determine whether this loss of activity was due to steric intolerance at the 4'-position, compounds 6a and 7a with methoxy groups at the 3'- and 2'-positions, respectively, were designed.

Target compounds 1a–7a can be obtained via the synthetic procedure used for lead compounds 1 and 2. However, the synthesis of 1 and 2 involved seven steps from commercial starting materials and the overall yield was a mere 6%. Hence, it was important to devise an efficient route for the synthesis of the tricyclic target compounds.
Such an efficient synthetic route could also be utilized in the synthesis of lead compounds 1 and 2, which are required in gram quantities for preclinical studies.

A.2 Design of 4-substituted 5-methyl-furo[2,3-d]pyrimidines as single agents with dual tubulin and RTK inhibitory potential

Figure 27. Lead compounds 181 and 182.

Gangjee et al. reported the 5-methyl-furo[2,3-d]pyrimidines 181 and 182 (Figure 27) with variation at N4-position.

Table 2. Effects of 181 and 182 on cell proliferation, microtubule depolymerization and [3H]-colchicine binding

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC50 ± SD (MDA-MB-435)</th>
<th>EC50 for MT depolymerization</th>
<th>Inhibition of colchicine binding (% inhibition ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 µM</td>
</tr>
<tr>
<td>181</td>
<td>&gt;10 µM</td>
<td>&gt;40 µM</td>
<td>-</td>
</tr>
<tr>
<td>182</td>
<td>4.3 ± 0.3 nM</td>
<td>23.9 nM</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>CA4</td>
<td>3.4 ± 0.6 nM</td>
<td>13.0 nM</td>
<td>88 ± 2</td>
</tr>
</tbody>
</table>

N4-Desmethyl 181 was inactive in tubulin assay and in MDA-MB-435 cells (Table 2). On the other hand, the N4-methyl analog 182 showed potent microtubule depolymerization and inhibition of [3H]-colchicine binding comparable to CA4.174
Table 3. RTK inhibitory activity of 181 and 182

<table>
<thead>
<tr>
<th>Compd</th>
<th>EGFR inhibition (nM)</th>
<th>VEGFR-2 inhibition (nM)</th>
<th>PDGFR-β inhibition (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>181</td>
<td>283.1 ± 40.1</td>
<td>38.1 ± 4.2</td>
<td>57.3 ± 8.0</td>
</tr>
<tr>
<td>182</td>
<td>15.5 ± 2.0</td>
<td>9.3 ± 0.72</td>
<td>12.3 ± 2.0</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
<td></td>
</tr>
</tbody>
</table>

In the kinase inhibition assays, the \(^{N^4}\)-methyl analog 182 was more active than the NH analog 181 (Table 3).\(^{175}\) More importantly, compound 182 was 2-fold and 7-fold more active than sunitinib in the VEGFR-2 and PDGFR-β kinase assays, respectively.

![Chemical Structures](image)

**Figure 28.** Conformational restriction of \(C_4-N\) and \(N-C_1'\) bonds due to \(^{N^4}\)-methyl group.

Compared to the NH analog, the \(N\)-methyl compound 182 showed spectacular antimitotic and antitumor activities, which could be attributed, in part, to conformational restriction of the bonds connecting the pyrimidine ring and the phenyl ring (Figure 28). In 181, the \(C_4-N\) and \(N-C_1'\) bonds connecting the pyrimidine and the phenyl moieties are freely rotatable. Whereas in 182, these bonds are somewhat restricted due to the presence of steric bulk of \(^{N^4}\)-methyl group and/or due to steric clash between the \(^{N^4}\)-methyl and 5-methyl groups (Figure 28).
Figure 29. The conformations and $^1$H NMR analyses of 181 (Fig. A) and 182 (Fig. B).
The $^1$H NMR spectra of the $N^4$-desmethyl analog $181$ and the $N^4$-methyl analog $182$ provided valuable information related to conformational restriction in $182$ (Figure 29).$^{175}$ For $181$, the “5-Me” protons appear at $\delta = 2.40$ ppm whereas for $182$, they are significantly shielded at $\delta = 1.05$ ppm. This shielding of the “5-Me” protons in $182$ is due to the proximity of a diamagnetic anisotropic cone in $182$ arising from the phenyl ring. The steric bulk of the $N^4$-methyl and/or steric clash between the $N^4$-methyl and 5-methyl groups in $182$ restrict the conformation and thus position the phenyl group on top of the 5-methyl group (as in Figure 29B) resulting in the shielding effect.

![Figure 30](image)

**Figure 30.** Series II.

Compounds $1b$–$4b$ (Series II, Figure 30) with varying alkyl groups at the $N^4$-position were designed to determine the steric tolerance at the $N^4$-position in the colchicine-binding site of tubulin and/or the ATP-binding site of RTKs. In addition, the larger alkyl groups in $1b$–$4b$ require greater volume and further restrict the free rotation of the C$_4$-N and N-C$_1'$ bonds. Introduction of these alkyl groups could improve the RTK-inhibitory activity of $182$ by forming additional hydrophobic interactions in the binding site (Figure 31).
Figure 31. Predicted binding modes of 182 (R = Me) in the ATP-binding site and docked poses of 182 in VEGFR-2 (PDB: 2XIR).\cite{176}

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 utilized by ATP for binding. The furo[2,3-\textit{d}]pyrimidine ring of \textbf{182} and the designed compounds \textbf{1b–4b} could bind to the Adenine region similar to heterocyclic scaffold inhibitors such as quinazolines and pyrrolo[2,3-\textit{d}]pyrimidines.\textsuperscript{177} If the furo[2,3-\textit{d}]pyrimidines (exemplified by \textbf{182}) occupy binding mode 1 (Figure 31), alkylation at \textit{N}\textsuperscript{4}-position might not be sterically tolerated. Alternatively, the furo[2,3-\textit{d}]pyrimidines could adopt binding mode 2 in which the compounds are rotated around the 2-\textit{H}–\textit{C}\textsubscript{2} bond. In this mode, the \textit{N}-alkyl group could orient towards the Sugar binding pocket or Hydrophobic region II and the 4-anilino group could occupy Hydrophobic region I. These compounds could also adopt binding mode 3 in which the molecule is rotated by 60° (from mode 2). In this mode, the \textit{N}-alkyl and the 4-anilino groups could occupy the Sugar binding pocket and Hydrophobic region I, respectively. Docking studies performed on the X-ray crystal structure of VEGFR2 (pdb: 2XIR)\textsuperscript{176} suggested the possibility of several binding modes. Representative low energy binding modes (\textbf{182} (R = Me) makes a hydrogen bond interaction with the backbone NH of cysteine in the hinge region.

\textbf{Figure 32}. Series III.

Compounds \textbf{1c–5c} (Series III, Figure 32) were designed to determine the importance of the 4'-methoxy group for tubulin and RTK inhibition. Compounds \textbf{1c}, \textbf{2c} and \textbf{4c} were designed to examine the role of 4'-methoxy group as a hydrogen-bond acceptor.
Compound 3c with a 4'-NHMe group was designed to determine if a hydrogen-bond donor is tolerated at this position. Compound 5c containing the 4'-NO₂ group was designed to know whether an electron-withdrawing group is tolerated at this position for RTK inhibitory activity. On the basis of the previous SAR on related pyrimidine-fused scaffolds, compound 5c is not expected to have antitubulin activity.

Gram-scale synthesis of 182 was required for further preclinical evaluations including toxicity and xenograft studies in mice.

**B. Inhibition of tubulin**

Tubulin binding agents disrupt microtubule dynamics leading to mitotic arrest and cell death. Half of all human tumors have mutations in the p53 gene, and the most effective drugs in p53 mutant cell lines are tubulin binding agents. Tubulin binding agents such as taxanes and vinca alkaloids are widely used for the treatment of solid tumors and hematological malignancies.

However, the enormous clinical success of taxanes and vinca alkaloids has been compromised by two major mechanisms of tumor resistance: Overexpression of Pgp and the expression of the βIII-tubulin. Pgp overexpression is clinically observed in many tumor cell lines, particularly in patients who have received chemotherapy. Overexpression of Pgp resulted in poor response to taxol-based chemotherapy in patients with non-small cell lung cancer. Use of Pgp inhibitors in overcoming Pgp-mediated resistance was not successful due to intolerable side effects. Tubulin binding agents that are not substrates of Pgp (for e.g. epothilones) represent a viable alternate strategy for circumventing Pgp-mediated resistance. Such agents would be extremely useful for patients that develop resistance due to Pgp overexpression.
The expression of βIII-tubulin is involved in clinical resistance to taxanes and Vinca alkaloids in non-small cell lung,\textsuperscript{45, 46, 52} breast,\textsuperscript{47} ovarian\textsuperscript{48, 53} and gastric\textsuperscript{49} cancers. Colchicine-site binding agents were not susceptible to βIII-tubulin mediated resistance,\textsuperscript{54, 55} which demonstrates the importance of developing anticancer drugs that bind to the colchicine-site. Although there are no colchicine-site agents in the clinic, several agents including CA4P (Fosbretabulin®)\textsuperscript{25-28} and CA1P (OXi4503)\textsuperscript{29} are currently in phase 1 and 2 clinical trials. Development of tubulin binding agents that are less sensitive to Pgp and/or βIII-tubulin mediated resistance could result in broader antitumor activity and improved rates of survival.

B.1 Design of $N^4$-aryl-5-chloro-2,4-diamino-pyrimido[4,5-$b$]indoles as inhibitors of tubulin

![Figure 33. Tubulin inhibitor 183.](image)

Pyrimido[4,5-$b$]indole 183 (Figure 33) is a potent microtubule depolymerizing agent that inhibited the growth of cancer cells with GI\textsubscript{50} values in the submicromolar range.\textsuperscript{182} It was discovered to be a colchicine-site binding agent and also overcame the Pgp and βIII-tubulin mediated drug resistance clinically observed with paclitaxel and vinca alkaloids.
Table 4. Compound 183 inhibits cell proliferation and tubulin assembly

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC\textsubscript{50} ± SD (MDA-MB-435)</th>
<th>Inhibition of tubulin assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>183</td>
<td>183 nM</td>
<td>7.1 ± 0.8 µM</td>
</tr>
<tr>
<td>CA4</td>
<td>3.4 nM</td>
<td>1.0 ± 0.09 µM</td>
</tr>
</tbody>
</table>

Compound 183 had a 7-fold lower inhibition of tubulin assembly than the standard colchicine site agent CA4 (Table 4).\textsuperscript{182} It showed a 60-fold higher IC\textsubscript{50} value than CA4 in MDA-MB-435 cancer cell lines. Compound 183 represented the first example of pyrimido[4,5-b]indoles as a potent tubulin inhibitor. It was of interest to identify the structural features of the tricyclic pyrimido[4,5-b]indoles that contribute to antitubulin activity. Hence, compounds 1d–6d (Series IV, Figure 34) were designed as potential inhibitors of tubulin.\textsuperscript{182}

\textbf{Figure 34. Series IV.}

Compound 1d (Figure 34) was designed to determine the importance of the 4'-methoxy group to tubulin inhibition. Compounds 2d and 3d are designed to evaluate the role of electron-donating and electron-withdrawing groups at 4'-position. The 3'-methoxy analog 4d was designed to explore the effect of a methoxy group at this position to
antitubulin activity. Compound 5d was designed to evaluate the effect of electron-withdrawing group at the 3'-position.

Compound 6d (Figure 34) was designed as a conformationally restricted analog by incorporating the bicyclic 6-methoxy-tetrahydroquinoline moiety onto the 4-position of the pyrimido[4,5-b]indole. Compared to the N-methylanilines, the tetrahydroquinoline moiety eliminates the rotation around the “N-Ph” bond thereby restricting the conformation of the phenyl ring. The restricted confirmation of the phenyl group in 5d results in a much more rigid structure than 183 but still maintains the phenyl and alkyl substitutions on the N4-position as in 183.

B.2 Design of 2,4-substituted pyrimido[4,5-b]indoles as inhibitors of tubulin

![Chemical structure](image)

**Figure 35.** Pyrimido[4,5-b]indoles 184 and 185.

Gangjee et al.\textsuperscript{178} reported the pyrimido[4,5-b]indoles 184 and 185 (Figure 35) which vary in substitution at the N4-position. The 4-NH analog 184 was inactive in MDA-MB-435 cell lines and in the microtubule depolymerization assay (Table 5). The N-methyl analog 185 is a colchicine-site agent, which inhibited cell proliferation about 4-fold less than CA4. Also, compound 185 circumvented clinically relevant mechanisms of tumor resistance of paclitaxel due to Pgp and βIII-tubulin.
Table 5. Effects of 184 and 185 on proliferation of MDA-MB-435 cells, microtubule depolymerization and \[^3\text{H}\]-colchicine binding

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC(_{50}) ± SD (MDA-MB-435)</th>
<th>EC(_{50}) microtubule depolymerization</th>
<th>Inhibition of colchicine binding (% inhibition ± SD) at 5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>184</td>
<td>&gt;40 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>185</td>
<td>14.7 ± 1.5 nM</td>
<td>105 ± 12 nM</td>
<td>84 ± 0.5</td>
</tr>
<tr>
<td>CA4</td>
<td>3.4 ± 0.6 nM</td>
<td>13.0 nM</td>
<td>99 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 36. Series V.

Compounds 1e–3e (Figure 36) with variations at \(N^4\)-position were proposed to improve the tubulin inhibitory activity of 185. Compound 1e with a larger ethyl group at the \(N^4\)-position was designed to determine the effect of size at the \(N^4\)-position on antitubulin activity. Compound 2e contains a 6-methoxy-tetrahydroquinoline moiety at the 4-position of the pyrimido[4,5-b]indole. It was designed to evaluate the effect of conformational restriction of the phenyl group on tubulin inhibition. The 4'-ethoxy analog 3e was designed based on molecular modeling (Figure 37).
Figure 37. Stereoview. Superimposition of the docked conformation of 3e (white) overlaid with colchicine (red) in the colchicine-site of tubulin (PDB: 1SA0)\textsuperscript{183}. Figure 37 shows the docked conformation\textsuperscript{178} of 3e (-7.75 kcal/mol) in the colchicine site of tubulin (PDB: 1SA0)\textsuperscript{183} obtained using MOE 2013.08.\textsuperscript{184} The oxygen atom of the 4’-OEt group of 3e forms hydrogen bonding with Cys\(\beta\)241 of tubulin. Also, the 4’-OEt group forms hydrophobic interactions with Val\(\alpha\)318 and Ile\(\beta\)378, which were absent in colchicine and 185. Compound 3e was designed based on the hypothesis that the replacement of 4’-OMe of 185 with the larger 4’-OEt group would allow additional interactions with the hydrophobic residues in the colchicine-site of tubulin.

Compound 1f (Figure 38) is designed by the bioisostere replacement of the 4’-OMe group in 185 with 4’-SMe group. This modification was found to improve antitubulin activity in the cyclopenta\([d]\)pyrimidines\textsuperscript{185} and the furo[2,3-\(d\)]pyrimidines.\textsuperscript{186}
Compounds 2f and 3f (Figure 38) with 2-Me and 2-H substitutions are designed to evaluate the importance of the 2-NH<sub>2</sub> group in antitubulin activity. These substitutions are well tolerated and afforded increased antitubulin activity in pyrimidine-fused bicyclic systems.<sup>185, 186</sup>

Bulk syntheses of potent tubulin inhibitors 183 and 185 were carried out for further preclinical evaluations.

C. Selective inhibition of <i>T.gondii</i> thymidylate synthase

Infection by the parasite <i>T. gondii</i> can lead to toxoplasmosis in immune compromised patients such as organ transplant, patients undergoing chemotherapy for cancer and HIV/AIDS patients.<sup>72</sup>

**Figure 39.** Representative drugs in the treatment of <i>T. gondii</i> infection.

Standard therapy for the treatment of toxoplasmosis involves a combination of sulfa drugs like sulfadiazine (Figure 39) and DHFR inhibitors such as pyrimethamine along
with folinic acid for rescue of bone marrow toxicity caused by pyrimethamine. Pyrimethamine selectively targets *T. gondii* DHFR where as sulfadiazine inhibits dihydropteroate synthase (DHPS) which catalyzes the *de novo* folate biosynthesis, a pathway absent in humans. The current therapies are limited by drug intolerance, central nervous system (CNS)-related side effects associated with sulfonamides and treatment failures owing to resistance. Pyrimethamine with clindamycin therapy may be used an alternative in patients allergic to sulfa drugs. Although this therapy resulted in lower allergic reactions than pyrimethamine-sulfadiazine therapy, 30% of patients did not respond to the treatment. Drug resistance and treatment failures to these very similar first-line agents indicate an urgent need for new agents.

The TS–DHFR enzyme is crucial for dTMP synthesis in *T. gondii* and hence represents a valid target to combat *T. gondii* infection. However, inhibitors selective for *T. gondii* TS (tgTS) over human TS (hTS) are not known at the time this research project was conceived. This is mainly due to the significant homology between the active site residues of tgTS and hTS. Availability of selective tgTS inhibitors would avoid the aforementioned drawbacks associated with current therapies.

**C.1 Synthesis of 2-amino-4-oxo-5-thioaryl-9H-pyrimido[4,5-b]indoles as selective inhibitors of *T. gondii* thymidylate synthase**

![Chemical structure](image)

**Figure 40.** 2-Amino-4-oxo-pyrimido[4,5-b]indoles 1g–7g (Series VII).
Gangjee et al.\textsuperscript{192} serendipitously discovered 2-amino-4-oxo-5-arylthio-pyrimido[4,5-b]indoles 1g–6g (Figure 40) as selective inhibitors of tgTS. Remarkably, compounds 1g–4g displayed 10-fold selectivity for tgTS over hTS compared to 0.2- and 3-fold selectivities for clinically used raltitrexed and pemetrexed, respectively (Table 6). The 5-thionaphthyl analogs 2g and 4g had low two-digit nanomolar tgTS inhibitory activities.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Human TS (µM) (A)</th>
<th>T. gondii TS (µM) (B)</th>
<th>Selectivity Ratio (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1g</td>
<td>2.7</td>
<td>0.13</td>
<td>20.8</td>
</tr>
<tr>
<td>2g</td>
<td>0.21</td>
<td>0.012</td>
<td>17.5</td>
</tr>
<tr>
<td>3g</td>
<td>1.3</td>
<td>0.13</td>
<td>10</td>
</tr>
<tr>
<td>4g</td>
<td>0.27</td>
<td>0.027</td>
<td>10</td>
</tr>
<tr>
<td>5g</td>
<td>0.75</td>
<td>0.23</td>
<td>3.3</td>
</tr>
<tr>
<td>6g</td>
<td>1.8</td>
<td>0.65</td>
<td>2.7</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.38</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Pemetrexed</td>
<td>9.5</td>
<td>2.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Encouraged by the preliminary data, additional quantities of 1g–6g were required for further preclinical evaluation including X-ray crystal and T. gondii culture studies and for pharmacokinetic studies. Compounds 1g–6g have been previously synthesized albeit in poor yields (2–6%) from commercially available starting materials.\textsuperscript{192} The unprecedented tgTS selectivity of 1g–6g (Table 6) prompted the design of an efficient synthetic strategy for preparing these compounds. This synthetic strategy can also be utilized for preparing novel analogs related to Series VII. Compound 7g is one such analog which was designed based on molecular modeling (Figure 41).
Figure 41. Compounds 1g and 7g in the active site of tgTS. (A) Surface map of tgTS active site occupied by 1g (red) and 7g (yellow) (PDB: 4KY4)\textsuperscript{193}. Hydrophobic and polar surfaces are colored green and magenta, respectively. (B) Key interactions between tgTS residues and 1g or 7g.

Compounds 1g and 7g were docked in the X-ray crystal structure of tgTS (PDB: 4KY4)\textsuperscript{193} using LeadIT.\textsuperscript{194} The 5-thiophenyl substitution of 1g accesses a hydrophobic pocket formed by Ile402, Leu516, Phe374 and Phe530 (Figure 41A). Since the phenyl ring does not occupy the large hydrophobic pocket, it was of interest to append a 4'-phenoxy group in target compound 7g in an attempt to gain additional interactions with Phe374 and therefore improve the tgTS activity. Figure 41B provides key interactions made by 1g and 7g in the active site of tgTS. The 2-NH\textsubscript{2} of both 1g and 7g forms hydrogen bonding with Ala609 and Asp513 of tgTS whereas the 3-NH forms hydrogen bonding with the Asp513. The tricyclic scaffold is stabilized by hydrophobic interaction with Leu516 and Trp403. Compound 7g forms additional hydrophobic interactions with Ile402, Phe374 and Phe520 and hydrogen bonding (indole NH) with Asn406. Also, the 4'-phenoxy 7g would probe the hydrophobic pocket and thus help us know whether hydrophobic residues are involved in selectivity for tgTS over hTS.
Compounds 1g–6g had poor water solubility and thus, to improve water-solubility, HCl salts of two most tgTS selective compounds, 1g and 2g, were also synthesized.
IV. CHEMICAL DISCUSSION

Synthesis of 5-thioaryl-9H-pyrimido[4,5-b]indole-2,4-diamines 1a–7a and lead compounds 1 and 2

Target compounds 1a–7a can be synthesized utilizing the synthetic procedure for lead compounds 1 and 2. However, the synthesis of 1 and 2 from commercially available 1,2-dichloro-3-nitrobenzene required seven steps and had a mere 6% overall yield.\textsuperscript{125} Hence, it was important to devise an efficient high-yielding strategy for the synthesis of target compounds 1a–7a. It was our intention that the synthetic scheme devised should also be adaptable to gram-scale synthesis of lead compounds 1 and 2 for preclinical evaluation.

\textbf{Figure 42.} Retrosynthesis of target compounds 1a–7a.

The synthesis of target compounds 1a–7a was envisioned via a nucleophilic displacement reaction\textsuperscript{125} of 5-chloro- or 5-bromo-pyrimido[4,5-b]indoles of general structure A (Figure 42), which could be prepared by condensation\textsuperscript{125} of substituted
indoles B with chlorformamidine. Indoles B can in turn be synthesized via reductive cyclization\textsuperscript{125} of appropriately substituted nitrobenzenes C, which could be obtained by electrophilic aromatic substitution\textsuperscript{125} of 1,2-dichloro- or 1,2-dibromo-3-nitrobenzene D with malononitrile.

In an effort to develop this method, initially the synthesis of lead compounds 1 and 2 was undertaken (Scheme 33), with the plan to extend the new scheme, if successful, to the target compounds 1a–7a, which contain electron-withdrawing or sterically demanding thioaryl groups at 5-position.

Scheme 33. Efficient gram-scale synthesis of lead compounds 1 and 2

Displacement of 1,2-dichloro-3-nitro-benzene 7 with the sodium salt of malononitrile anion provided 2-(2-chloro-6-nitrophenyl)malononitrile 186 (Scheme 33). Several reaction conditions such as varying the solvent (THF, DMF) and/or the base (potassium tert-butoxide, sodium hydride) and/or temperature (75 °C to 120 °C) were screened for this step. The reaction did not go to completion [monitored by thin–layer chromatography (TLC)] when potassium tert-butoxide was employed as the base. Using
THF as solvent also did not result in complete utilization of the reactant 7 even after 48 h. With DMF/NaH as the solvent/base combination, reactions were performed at temperatures of 75 °C, 100 °C and 120 °C. Optimum temperature was found to be 100 °C for improved yields and less side products (based on TLC).

Reductive cyclization of 186 using zinc dust in acetic acid furnished 2-amino-4-chloro-1H-indole-3-carbonitrile 187 (Scheme 33). The 1H NMR of 187 showed the presence of the indole NH peak at 10.98 ppm and the 2-NH2 peak at 6.91 ppm and both these peaks exchanged in the presence of D2O. This confirmed the formation of the indole 187 and interm indicated that the 2-chloro group in 7, and not the 3-chloro, was displaced by the malononitrile anion to provide 186. Cyclocondensation of 187 with carbamimidic chloride hydrochloride195 afforded the 2,4-diamino-5-chloro-pyrimido[4,5-b]indole 188125 in 45% yield. The reactant 187 was completely utilized after 2 h and two spots (1:1 ratio) were observed on TLC (5% MeOH in CHCl3). It was assumed that one of the products might be the uncyclized analog of 188. Upon prolonging the reaction time to 20 h, only one spot was observed on TLC indicating that the other spot initially observed was probably an uncyclized analog of 188.

This synthetic strategy offers an effective route for the synthesis of the valuable 5-chloro intermediate 188 from 1,2-dichloro-3-nitro-benzene 7. It provided 188 in three steps with an overall yield of 22% compared to the reported procedure125 which gave 188 in six steps with 5% overall yield. Compound 188 was then treated with aryl thiols 189 and 190 to provide 1 g of 1 and 2 respectively.125
Scheme 34. Attempted synthesis of target compound 1a

However, similar reaction of 188 with arylthiols containing electron-withdrawing substituents (e.g. 191) resulted in no S_{N}Ar reaction (e.g. 1a) (Scheme 34). This decreased reactivity can be attributed to the reduced nucleophilicity of these arylthiols due to the negative inductive effect associated with the electron withdrawing substituents that make the electrons on the sulfur less available for a nucleophilic attack. In addition to this, the 5-chloro group in 188 is not a good leaving group in the S_{N}Ar reaction first due to the absence of activation, and second due to the presence of an indole nitrogen meta to the chlorine which can donate electrons into the C-ring making it electron rich, and hence, making the 5-carbon less electrophilic. One of the ways to facilitate the reaction between 188 and 191 is to employ metal-catalyzed coupling.\textsuperscript{173, 196, 197} Copper-catalyzed coupling (CuI/K_{2}CO_{3}/DMF)\textsuperscript{173, 197} between 188 and 191 did not give any new product (based on TLC). Palladium-catalyzed coupling\textsuperscript{173, 196} between 188 and electron-withdrawing arylthiols such as 191 also failed (Scheme 34). Another alternative for improving the reactivity is by increasing the electrophilicity of the 5-carbon of 188, which can be
obtained by replacing the 5-chloro of 188 with a 5-bromo group. The bromo group at the 5-position is easier to displace than a chloro group because of its higher electronegativity.

**Scheme 35. Synthesis of target compounds 1a–6a**

Thus, synthesis of target compounds 1a–6a started from 2,3-dibromonitrobenzene 192 (Scheme 35) that was synthesized by nitration of 1,2-dibromobenzene (the major product is 1,2-dibromo-4-nitrobenzene; purification was accomplished by column chromatography). Displacement of the 2-bromo of 192 with malononitrile using sodium hydride provided 193. The use of potassium tert-butoxide resulted in only 60% utilization of 192. Reductive cyclization\textsuperscript{125} of 193 using zinc dust furnished 194, which upon cyclocondensation with carbamimidic chloride hydrochloride\textsuperscript{195} afforded the 2,4-diamino-5-bromo-pyrimido[4,5-b]indole 195. Ullmann coupling of 195 with appropriate arylthiols 191, 196–200 using CuI, K\textsubscript{2}CO\textsubscript{3} under microwave conditions furnished the target compounds 1a–6a in 21–64% yield. Column chromatography of 1a–6a should not
be carried out using a CombiFlash® Rf system because the compounds stick to the silica gel and do not elute out. Ullmann coupling of 195 and 201 using similar conditions resulted in partial utilization of the reactant 195 and/or formation of multiple side products (TLC). Hence, it was decided to perform Ullmann coupling either at lower temperatures or by using other copper catalysts (Scheme 36).

**Scheme 36. Synthesis of target compound 7a**

![Scheme 36](image)

Ullmann coupling of 195 and 201 (Scheme 36) at lower temperatures (100–145 °C) resulted in only 50–60% utilization of reactant 195 (based on TLC). As the strategy of coupling at lower temperatures did not work, the next option considered was to attempt other copper catalysts. Copper catalysts Cu₂O/CuBr have been successfully employed for Ullmann coupling in the synthesis of nolatrexed. Ullmann coupling of 195 and 201 using CuBr/Cu₂O resulted in 7a in 31% yield (Scheme 36).

**Synthesis of N,N-disubstituted-5-methylfuro[2,3-d]pyrimidin-4-amines 1b–4b and 1e–5e**

Target compounds 1b–4b were synthesized as described in Scheme 37. Compound 203 was obtained by reacting hydroxyacetone 202 with malononitrile in the presence of triethylamine and was used without purification for the next step. Treatment of 203
with formamidine (obtained by stirring formamidine hydrochloride with sodium ethoxide) in ethanol provided 4-amino-5-methyl-furo[2,3-d]pyrimidine 204.\textsuperscript{174} \textsuperscript{1}H NMR of 204 showed the presence of the 4-NH$_2$ as a broad singlet at 7.02 ppm and the C$_2$-CH as a singlet at 8.13 ppm. Ullmann coupling of 204 and 4-iodo anisole 205 using copper iodide and L-proline afforded 181 in 33% yield.\textsuperscript{174} N$^4$-Alkylation\textsuperscript{174} of 181 with alkyl iodides 206–209 furnished 1b–4b in 57-85% yield.

**Scheme 37. Synthesis of target compounds 1b–4b**

![Scheme 37. Synthesis of target compounds 1b–4b](image)

**Scheme 38. Synthesis of target compound 1c**

![Scheme 38. Synthesis of target compound 1c](image)
Ullmann coupling\(^{200}\) of 204 and \(p\)-ethyl phenyl iodide 210 (Scheme 38) using copper iodide and L-proline afforded 213 in 29% yield. Compound 213 was \(N^4\)-methylated using dimethyl sulfate to furnish target compound 1c in 53% yield. Ullmann coupling of 204 with aryl iodides 211 and 212 using copper iodide and L-proline provided 214 and 215 in poor yields. In these reactions, it was observed that 50-60% of 204 was unreacted (based on TLC). Using higher (2–3) equivalents of CuI and/or L-proline did not improve the reactivity of 204. The reactivity of 204 (or the reaction yield) also did not improve under higher reaction temperatures (120–150 °C) and/or microwave conditions. To improve the reactivity of 204 as well as the reaction yield, it was decided to change the ligand “L-proline” and/or the catalyst “copper iodide”. Guo, X. et al.\(^{201}\) successfully utilized pipecolinic acid as the ligand for \(N\)-arylation of electron-deficient aromatic systems. Hence, pipecolinic acid, instead of L-proline, was employed as the ligand for Ullmann coupling of 204 and aryl iodides 211 or 212 (Scheme 39).

**Scheme 39. Synthesis of target compounds 2c–5c**
Ullmann coupling\textsuperscript{201} of 204 with aryl iodides 211 and 212 using pipecolinic acid as the ligand provided significantly better yields for 214 and 215 (Scheme 39), respectively, than when L-proline was the ligand. Similarly, treatment of 204 with \textit{p}-iodo anilines 216\textsuperscript{202} and 217\textsuperscript{202} afforded 218 and 219, respectively in moderate yields. \textit{N}^4-Methylation of 214, 215 and 219 using dimethyl sulfate at room temperature afforded target compounds 2c, 5c and 4c, respectively, in 43-51% yield. Similar conditions for the \textit{N}^4-methylation of 218 resulted in the methylation of both 4- and 4'-nitronegens to give the 4'-NMe\textsubscript{2} analog 4c instead of desired compound 3c. Reaction at rt or the use of more than one equivalent of NaH gave predominantly 4c. Hence, compound 218 was treated with NaH (1.1 equivalents) and dimethyl sulfate (1.1 equivalents) at -10–0 °C to furnish the desired compound 3c in 20% yield and 4c in 30% yield.

**Retrosynthetic strategies for 2,4,5-trisubstituted pyrimido[4,5-\textit{b}]indoles**

\textbf{Figure 43.} Retrosynthetic strategies for target compounds 1d–6d, 1e–3e and 1f–3f.
The synthesis of target compounds 1d–6d, 1e–3e and 1f–3f was envisioned via nucleophilic displacement reactions\textsuperscript{125} of 4-chloro-pyrimido[4,5-b]indoles with general structure A (Figure 43), which could be prepared from 4-oxo- pyrimido[4,5-b]indoles B. Compounds of general structure B can be synthesized using either of the two strategies. Strategy 1 involves condensation\textsuperscript{125} of substituted indoles D with chlorformamidine type reagents C. Indoles D can be synthesized via reductive cyclization\textsuperscript{125} of appropriately substituted nitrobenzenes E, which could be obtained by treating\textsuperscript{125} o-chloro-nitrobenzenes D with malononitrile. Alternatively, 4-oxo- pyrimido[4,5-b]indoles B can be prepared via strategy II that utilizes a Fischer indole synthesis of G from aryl hydrazines H and cyclohexanones I.

**Synthesis of N\textsuperscript{4}-aryl-5-chloro-2,4-diamino-pyrimido[4,5-b]indoles 1d–6d**

Disconnection strategy I (Figure 43) was utilized for the synthesis of target compounds 1d–6d. Displacement of the 2-chloro group of 1,2-dichloro-3-nitro-benzene 7 by the ethyl cyanoacetate anion provided 8\textsuperscript{125} as a viscous yellow liquid in 96\% yield (Scheme 40). Reduction of the nitro group of 8 followed by cyclization furnished 9\textsuperscript{125} as a pink solid in 74\% yield.\textsuperscript{125} \textsuperscript{1}H NMR indicated the formation of the indole 9: a one-proton singlet at 10.97 ppm (indole NH) and a two-proton broad singlet at 6.87 ppm (2-NH\textsubscript{2} group) and both these peaks exchanged in the presence of D\textsubscript{2}O. Cyclocondensation of 9 with carbamimidic chloride hydrochloride\textsuperscript{195} afforded the tricyclic compound 10\textsuperscript{125} after workup as a pale brown solid in 64\% yield. Protection of the 2-amino group of 10 using pivalic anhydride provided 220\textsuperscript{125} in 60\% yield, better than the reported method\textsuperscript{125} which utilized pivalic anhydride, 4-(N,N-dimethylamino)pyridine (DMAP) and triethylamine to give 220 in 40\% yield. The
pivaloyl protection of the 2-amino group increases the solubility of the compound in organic solvents for the chlorination step; a direct chlorination of unprotected 10 with phosphorus oxychloride resulted in no reaction.

Scheme 40. Synthesis of target compounds 1d–6d

![Scheme 40](image)

Compound 221\textsuperscript{125} was obtained by treating 220 with phosphorus oxychloride (Scheme 40). Displacement\textsuperscript{203} of the 4-chloro group in 221 with N-methyl anilines 222–226 or 6-methoxy-1,2,3,4-tetrahydroquinoline 227 followed by base-mediated deprotection of the 2-amino group provided target compounds 1d–6d respectively in 52–67% yield.\textsuperscript{182} The amines 222–227 regiospecifically displaced the 4-chloro of 221

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rather than the 5-chloro group because the 4-position is more electrophilic than the 5-position owing to the presence of the adjacent 3-nitrogen atom.

**Synthesis of 2,4-disubstituted-9H-pyrimido[4,5-b]indole-4-amines 1e–3e and 1f–3f**

Target compounds 1e–3e and 1f–3f were synthesized by employing the disconnection strategy II (Figure 43), which involves a “Fisher indole synthesis” as the key step in the construction of pyrimido[4,5-b]indole ring.

**Scheme 41. Synthesis of target compounds 1e and 2e**
Displacement of the 6-chloro of 2-amino-6-chloropyrimidin-4(3H)-one 228 with hydrazine using a reported method\textsuperscript{204} afforded 229 in 46% yield (Scheme 41). Fischer indole cyclization of the 6-hydrazinopyrimidin-3-one 229 with cyclohexanone 230 furnished the tricyclic scaffold 231.\textsuperscript{178} This reaction required reflux in diphenyl ether (250 °C) to facilitate the cyclization to 231. The $^1$H NMR of 231 showed the presence of a D$_2$O-exchangeable indole NH peak at 10.50 ppm and four pairs of aliphatic CH$_2$ peaks at 1.64–1.69, 2.42 and 2.53 ppm. This structural data confirmed the formation of the indole 231. The partially saturated ring in 231 was oxidized using 10% Pd/C to provide 232\textsuperscript{178} in 57% yield. The 2-amino moiety of 232 was pivaloyl protected to give 233,\textsuperscript{178} which upon chlorination afforded the common intermediate 234.\textsuperscript{178} Attempts to displace the 4-chloro of 234 with anilines 235\textsuperscript{205} and 6-methoxy-1,2,3,4-tetrahydroquinoline 227 in isopropanol at reflux were unsuccessful. After 72 h, less than 5% of 234 was utilized (based on TLC). Hence, $n$-butanol, that has higher boiling point than isopropanol, was chosen as the solvent for the synthesis of 1e and 2e. Compound 234 was treated with 235 and 227 in $n$-butanol to afford 1e\textsuperscript{178} and 2e, respectively, after simultaneous deprotection of the 2-amino group.

**Scheme 42.** Synthesis of target compound 3e
Compound 3e was obtained from nucleophilic displacement of 234 with 4-ethoxy-N-methylaniline 236 and simultaneous deprotection of the 2-amino group (Scheme 42). Unlike the displacement of the 4-chloro of 234 with 235 and 227 (Scheme 41) which required heating in n-butanol at reflux, the displacement of 234 with 236 worked in isopropanol at reflux. This can be attributed to lower reactivity of the sterically hindered amines 235 and 227 compared to 236.

Scheme 43. Synthesis of target compound 1f

![Scheme 43](image)

Displacement of the 4-chloro of 234 with 4-thiomethyl-N-methylaniline 237 followed by simultaneous deprotection of the 2-amino group in n-butanol at reflux provided 1f in 72% yield (Scheme 43). n-Butanol was chosen as the solvent for 1f because the reaction of 234 with 237 in isopropanol at reflux resulted in less than 5% completion after 72 h (based on TLC).

The synthesis of target compound 2f commenced from commercially available 1-chloro-2-nitrobenzene 238 (Scheme 44). Displacement of the chloro of 238 by the ethyl cyanoacetate anion provided 239 in 86% yield. Reduction of the nitro group of 239 followed by cyclization furnished 18 in a yield of 74%. Bubbling anhydrous HCl gas through a solution of 18 in acetonitrile afforded 21. Compound 21 was then treated with aqueous NaOH solution in ethanol at reflux to obtain 23. Proton NMR of 23
confirmed the formation of 2-methyl-4-oxo-pyrimido[4,5-b]indole: a three-proton singlet (2-Me) at 2.41 ppm, two D$_2$O-exchangeable singlets (3-NH and 9-NH) at 11.98 ppm and 12.12 ppm and 4 aryl protons (between 7.18 and 7.94 ppm). Compound 23 was chlorinated\textsuperscript{178} using phosphorus oxychloride to afford 240 in 79% yield. Displacement of the 4-chloro of 240 with 4-methoxy-$N$-methyl aniline 241 provided target compound 2f in 78% yield.

**Scheme 44. Synthesis of target compound 2f**

The target compound 3f was synthesized using Scheme 45. 2-Amino-indole-3-nitrile 18 (Scheme 44) was reacted with neat formamide to afford pyrimido[4,5-b]indole 242 (Scheme 45).\textsuperscript{129} The 4-oxo of 242 was converted to the 4-chloro 243 by treating with phosphorus oxychloride. Nucleophilic displacement of 243 with 4-methoxy-$N$-methyl aniline 241 provided target compound 3f in 38% yield.
Scheme 45. Synthesis of target compound 3f

Synthesis of 2-amino-4-oxo-5-thioaryl-9H-pyrimido[4,5-b]indoles 1g–7g

Scheme 46. Synthesis of 1g–6g and novel compound 7g

Compounds 1g–6g have been previously synthesized albeit in very poor yields (2–6%) from commercial starting materials. To improve the yield, Ullmann coupling of 10 with aryl thiols 244–249 was attempted with copper iodide as the catalyst (Scheme 46). Several temperatures (145–200 °C), solvents (DMF, NMP) with/without ligand (L-proline) were screened. The optimal method is described in Scheme 46. Compound 10 was obtained in 45% yield from commercial starting materials in three steps (Scheme
40). Ullmann coupling of 10 with 244–246, 248–250 using CuI and L-proline at 180 °C under microwave conditions provided 1g–3g and 5g–7g in 47-62% yield. Similar displacement of 10 with 1-naphthyl thiol 247 was unsuccessful probably due to steric hindrance. Column chromatography of 1g–3g and 5g–7g should not be carried out with a CombiFlash® Rf system because the compounds stick to the silica gel and do not elute out. Optimization of this reaction resulted in the synthesis of 1g–3g, 5g and 6g in 23–28% overall yield from commercial starting materials, which is a significant improvement over the previous method which gave a mere 2–6% yield.

Scheme 47. Synthesis of 1g·HCl and 2g·HCl

Hydrochloride salts 1g·HCl and 2g·HCl were synthesized to improve the water solubility of 1g and 2g (Scheme 47). The HCl salt of 1g (1g·HCl) was obtained by bubbling anhydrous HCl gas through a solution of 1g in chloroform, ethyl acetate, diethyl ether and methanol. The HCl salt of 2g (2g·HCl) was prepared by treating 2g in DMF, dioxane and diethyl ether with anhydrous HCl gas.
V. EXPERIMENTAL

All evaporations were carried out in vacuum with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mm Hg) in a CHEM-DRY drying apparatus over P₂O₅ at 50 °C. Thin–layer chromatography (TLC) was performed on Whatman® Sil G/UV254 silica gel plates and the spots were visualized by irradiation with ultraviolet light (254 and 366 nm). 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 a 70–230 mesh silica gel (Fisher Scientific) column. Flash chromatography was carried out using CombiFlash® Rf 200 (Teledyne ISCO) automated flash chromatography system with pre-packed RediSep® Rf normal–phase flash columns (230–400 mesh). 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. Columns were wet-packed with appropriate solvent unless specified otherwise. Melting points were determined using a digital 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 Bruker Avance II 400 (400 MHz) and 500 (500 MHz) NMR systems and were analyzed using MestReC NMR data processing software. The chemical shift (δ) values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet; exch, protons exchangeable by addition of D₂O.

Elemental analyses or high-performance liquid chromatography (HPLC)/mass analysis were used to determine the purities of the target compounds. Elemental analyses were
performed by Atlantic Microlab, Inc., Norcross, GA. Elemental compositions are within ±0.4% of the calculated values and indicate >95% purity. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented despite 24–48 h of drying in vacuo and were confirmed where possible by their presence in the $^1$H NMR spectra. Mass spectrum data were acquired on an Agilent G6220AA TOF LC/MS system using the nano ESI (Agilent chip tube system with infusion chip). HPLC analysis was performed on a Waters HPLC system using XSelect® CSH C18 column. Peak area of the major peak w.r.t other peaks was used to determine the purity. All solvents and chemicals were purchased from Sigma–Aldrich Co. or Fisher Scientific Inc. and were used as received.

2-(2-Chloro-6-nitrophenyl)malononitrile (186).

To an ice-cold solution of malononitrile (10.3 g, 156 mmol) in anhydrous DMF (500 mL) under argon atmosphere was added sodium hydride (3.75 g, 156 mmol). The resulting white suspension was stirred for 15 min and then 1,2-dichloro-3-nitrobenzene 7 (10 g, 52 mmol) was added. An orange color was observed within 2-3 minutes after adding 7. The mixture was stirred at 100 °C for 4 h. After cooling the reaction mixture, 200 mL H$_2$O was added, and the aqueous mixture was acidified to pH 2.0 with conc. HCl. The mixture was extracted with diethyl ether (500 mL × 3) and the combined organic phases were dried using anhydrous sodium sulfate and concentrated to give 186 as dark brown oil. TLC $R_f = 0.72$ (hexane/EtOAc, 1:1); $^1$H NMR (500 MHz, DMSO-$d_6$): δ = 6.22 (s, 1H, CH); 7.72–7.75 (m, 1H, Ar); 7.92–7.93 (m, 1H, Ar); 8.18–8.20 (m, 1H, Ar). This material was used directly for the next step without further purification.
2-Amino-4-chloro-1H-indole-3-carbonitrile (187).

A solution of 186 (1 g, 4.5 mmol) in glacial acetic acid (60 mL) was treated with 3 g of zinc dust. The mixture was stirred at 60 °C for 45 min and then recharged with zinc dust (1 g). After heating for another 105 min, the mixture was filtered through a pad of Celite. The pad was washed with 60 mL AcOH, the filtrate was concentrated and then neutralized with saturated Na₂CO₃ solution. The resulting precipitate was then purified by column chromatography, eluting sequentially with 0% and 1% MeOH in CHCl₃. Fractions containing the product (TLC) were pooled and evaporated to afford 490 mg (49%, two steps) of 187 as a pink solid. TLC Rₑᵥ₄ = 0.25 (CHCl₃/MeOH, 10:1 with 2 drops of conc. NH₃OH); mp 240.1–240.9 °C. ¹H NMR (500 MHz, DMSO-d₆): δ = 6.84–6.88 (m, 1H, Ar); 6.91 (br, 2H, 2-NH₂, exch); 6.92–6.94 (m, 1H, Ar); 7.07–7.09 (m, 1H, Ar); 10.98 (s, 1H, 1-NH, exch). Elemental analysis calculated (%) for C₉H₆ClN₃·0.09CH₃COCH₃: C, 56.56; H, 3.35; N, 21.35; Cl, 18.01. Found: C, 56.36; H, 3.39; N, 21.31; Cl, 17.94.

5-Chloro-9H-pyrimido[4,5-b]indole-2,4-diamine (188).

Compound 187 (0.8 g, 4.16 mmol), carbamimidic chloride hydrochloride¹⁹⁵ (0.8 g) and methyl sulfone (8 g, 10 equivalents) were taken in a 100 mL evaporation flask and was heated at 120 °C for 20 h. About 10 mL of ammonia in methanol was added to neutralize the reaction mixture. Solvent was removed under reduced pressure and the product was purified by column chromatography, sequentially eluting with 1% and 5% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford 0.57 g (58%) of 188 as a brown solid. TLC Rₑᵥ₄ = 0.53 (CHCl₃/MeOH, 5:1 with 2 drops of
conc. NH₄OH; mp 243.2–243.8 °C (lit.¹²⁵ 245.2–246.3 °C). ¹H NMR (400 MHz, DMSO-
-d₆): δ = 6.07 (s, 2H, 4-NH₂, exch); 6.80 (bs, 2H, 2-NH₂, exch); 7.06–7.13 (m, 2H, Ar);
7.21–7.23 (m, 1H, Ar); 11.49 (s, 1H, 9-NH, exch). ¹H NMR agreed well with the
literature reported¹²⁵ values.

**General procedure for the synthesis of 5-(substituted phenylthio)-9H-pyrimido[4,5-
b]indole-2,4-diamines 1 and 2.**

Compound 188 (50 mg, 1 equivalent), potassium carbonate (4 equivalents) and
appropriate aryl thiol (4 equivalents) were weighed and added to a Biotage® microwave
vial. N-methyl pyrrolidine-2-one (NMP, 3 mL) was added as solvent and the vial was
sealed. The reaction was run in a Biotage® initiator at very high absorption for 30
minutes at 250 °C. The reaction mixture was transferred to the top of a 15 g silica gel
column and was sequentially eluted with 1% and 3% methanol in chloroform. Fractions
containing the product (TLC) were pooled and evaporated to give the desired product
which was further purified by washing with diethyl ether.

**5-(Phenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (1).**

Using the general procedure described above, compound 188 (50 mg, 0.22 mmol) was
treated with benzene thiol 189 (95 mg, 0.86 mmol) to yield 60 mg (90%) of 1 as a brown
solid. TLC $R_f = 0.62$ (CHCl₃/MeOH, 5:1 with 2 drops of conc. NH₄OH); mp 251.1-252.0
°C (lit.¹²⁵ 251 °C). ¹H NMR (400 MHz, DMSO-d₆): δ = 6.02 (s, 2H, 4-NH₂, exch);
7.03–7.04 (m, 2H, Ar); 7.13–7.15 (m, 1H, Ar); 7.22–7.25 (m, 3H, Ar), 7.24 (s, 2H, 2-
NH₂, exch); 7.32–7.33 (m, 1H, Ar); 7.40–7.42 (m, 1H, Ar); 11.48 (s, 1H, 9-NH, exch).
Elemental analysis calculated (%) for C_{16}H_{13}N_{5}S\cdot0.47CH_{3}OH: C, 61.35; H, 4.65; N, 21.72; S, 9.95. Found: C, 61.32; H, 4.41; N, 21.66; S, 9.89. \textsuperscript{1}H NMR agreed well with the literature reported\textsuperscript{125} values.

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

Using the general procedure described above, compound 188 (50 mg, 0.22 mmol) was treated with \(p\)-toluene thiol 190 (95 mg, 0.86 mmol) to provide 59 mg (86\%) of 2 as a brown solid. TLC \(R_f = 0.60\) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp >250 °C (lit.\textsuperscript{125} >250 °C). \textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}$_6$): \(\delta = 2.20\) (s, 3H, CH\(_3\)), 6.03 (bs, 2H, 4-NH\(_2\), exch), 6.96–6.98 (m, 2H, Ar), 7.06–7.08 (m, 2H, Ar), 7.18–7.22 (m, 1H, Ar), 7.20 (bs, 2H, 4-NH\(_2\), exch), 7.29–7.31 (m, 1H, Ar), 7.37–7.39 (m, 1H, Ar), 11.47 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C\(_{17}\)H\(_{15}\)N\(_5\)S: C, 63.53; H, 4.70; N, 21.79; S, 9.98. Found: C, 63.13; H, 4.85; N, 21.51; S, 9.87. \textsuperscript{1}H NMR agreed well with the literature reported\textsuperscript{125} values.

2-Amino-4-bromo-1\(H\)-indole-3-carbonitrile (194).

To an ice-cold solution of malononitrile (4.76 g, 72 mmol) in anhydrous DMF (500 mL) under argon atmosphere was added sodium hydride (2.6 g, 104 mmol). Thus formed white suspension was stirred for 15 min and then 1,2-dibromo-3-nitrobenzene 192\textsuperscript{198} (10 g, 36 mmol) was added. Orange color was observed within 2-3 minutes after adding 192. The mixture was stirred at 100 °C for 4 h. After cooling the reaction mixture, 200 mL H\(_2\)O was added, and the resulting aqueous mixture was acidified to pH 2.0 with conc. HCl. The mixture was extracted with diethyl ether (500 mL \(\times\) 3) and then the combined
organic phases were dried using anhydrous sodium sulfate and concentrated to give (2-Bromo-6-nitropheryl)-malononitrile 193 as dark-brown oil. TLC $R_f = 0.42$ (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 6.18$ (s, 1H, CH); 7.62–7.66 (m, 1H, Ar); 8.07–8.09 (m, 1H, Ar); 8.19–8.21 (m, 1H, Ar). The material was used directly for the next step.

A solution of 193 (3.92 g, 14.6 mmol) in glacial acetic acid (500 mL) was treated with a single charge of zinc dust (6.84 g, 2 parts by weight). The mixture was stirred at 60 °C for 45 min, then recharged with zinc dust (3.92 g, 1 part by weight). After heating for another 105 min, the mixture was filtered through a pad of Celite. The pad was washed with glacial acetic acid, the filtrate was concentrated and then washed with saturated Na$_2$CO$_3$ solution. The precipitate was collected and then dissolved in methanol. Silica gel (5 g) was added to make the plug and was then purified by column chromatography, eluting sequentially with 0% and 1% MeOH in CHCl$_3$. The fractions containing the pure product (TLC) were pooled and evaporated to give 2.52 g of 197 as a pink solid. The overall yield from 192 to 194 was 60%. TLC $R_f = 0.25$ (CHCl$_3$/MeOH, 10:1 with 2 drops of conc. NH$_4$OH); mp 231.1–231.9 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 6.78–6.82$ (m, 1H, Ar); 6.89 (bs, 2H, NH$_2$, exch); 7.07–7.08 (m, 1H, Ar); 7.10–7.12 (m, 1H, Ar); 10.98 (s, 1H, NH, exch). Elemental analysis calculated (%) for C$_9$H$_6$BrN$_3$: C, 45.79; H, 2.56; N, 17.80; Br, 33.84. Found: C, 45.89; H, 2.59; N, 17.57; Br, 33.56.
5-Bromo-9H-pyrimido[4,5-b]indole-2,4-diamine (195).

A mixture of 2-amino-4-bromo-1H-indole-3-carbonitrile 194 (3 g, 12.7 mmol), carbamimidic chloride hydrochloride\textsuperscript{195} (1.6 g, 13.9 mmol) and methyl sulfone (30 g) was stirred and stirred at 120 °C for 8 h, after which it was recharged with carbamimidic chloride hydrochloride\textsuperscript{195} (1.6 g, 13.9 mmol) and stirred for another 14 h. The reaction mixture was cooled and 50 mL of ammonia in methanol was added to neutralize the reaction mixture. Solvents were evaporated and the crude mixture was purified by column chromatography, eluting sequentially with 1% and 5% methanol in chloroform. Fractions containing the desired product (TLC) were pooled and evaporated to afford 2.16 g (61%) of 195 as an off-white solid. TLC $R_f = 0.27$ (CHCl$_3$/MeOH, 10:1 with 2 drops of conc. NH$_4$OH); mp 239.8–240.1 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 6.05$ (bs, 2H, 2-NH$_2$, exch); 6.95 (br, 2H, 4-NH$_2$, exch); 7.01–7.05 (m, 1H, Ar); 7.24–7.26 (m, 2H, Ar); 11.49 (br, 1H, 9-NH, exch). HRMS (ESI): $m/z$ calculated for C$_{10}$H$_8$BrN$_5$ + H$^+$ [M+H$^+$]: 278.0036, 280.0016. Found: 278.0029, 280.0005.

General procedure for the synthesis of 5-(substituted phenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamines 1a–6a.

Compound 195 (1 equivalent), appropriate aryl thiol (4 equivalents), copper iodide (4 equivalents) and potassium carbonate (8 equivalents) were added to a Biotage\textsuperscript{®} microwave vial. DMF (3 mL) was added as the solvent and the vial was sealed. The reaction was run in a Biotage\textsuperscript{®} Initiator at 180 °C for 4.5–6.5 h (depending on the aryl thiol). After cooling to room temperature, silica gel (0.3 g) was added and DMF was removed under reduced pressure and the crude product was purified by column
chromatography, sequentially eluting with 1%, 2% and 3% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford the product.

**5-(4-Fluorophenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (1a).**

Using the general procedure described above, the reaction of 195 (40 mg, 0.14 mmol) with 4-fluorophenylthiol 191 (0.06 mL, 0.56 mmol), copper iodide (110 mg, 0.56 mmol) and potassium carbonate (159 mg, 1.12 mmol) for 6.5 hours afforded 33 mg (64%) of 1a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp 267.9–268.9 ºC. $^1$H NMR (400 MHz, DMSO-$d_6$): δ = 6.08 (s, 2H, 2-NH$_2$, exch), 7.11–7.41 (m, 9H, Ar and 4-NH$_2$), 11.52 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{16}$H$_{12}$FN$_5$S·0.26CH$_3$OH: C, 58.52; H, 3.94; N, 20.99; S, 9.61; F, 5.69. Found: C, 58.35; H, 3.85; N, 21.06; S, 9.84; F, 5.54.

**5-(4-Trifluoromethylphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (2a).**

Using the general procedure described above, the reaction of 195 (50 mg, 0.18 mmol), 4-trifluoromethylphenylthiol 196 (0.2 mL, 0.72 mmol), copper iodide (137.5 mg, 0.72 mmol) and potassium carbonate (198.75 mg, 1.44 mmol) for 6.5 hours afforded 32 mg (46%) of 2a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp >250 ºC. $^1$H NMR (400 MHz, DMSO-$d_6$): δ = 6.04 (s, 2H, 2-NH$_2$, exch); 7.12–7.29 (d, 4H, Ar); 7.35–7.48 (m, 3H, Ar and 4-NH$_2$); 7.59–7.61 (d, 2H, Ar); 11.57 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{17}$H$_{12}$F$_3$N$_5$S·0.32CH$_3$OH: C,
53.94; H, 3.47; N, 18.16; S, 8.32; F, 14.78. Found: C, 54.25; H, 3.32; N, 17.96; S, 8.32; F, 14.55.

5-(*m*-Tolylthio)-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine (3a).

Using the general procedure described above, the reaction of 195 (50 mg, 0.175 mmol) with *m*-toluene thiol 197 (0.09 mL, 0.7 mmol), copper iodide (137.5 mg, 0.7 mmol) and potassium carbonate (198.7 mg, 1.4 mmol) for 5.5 hours afforded 18 mg (31%) of 3a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp 228.0–228.5 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.19$ (s, 3H, 3'-Me), 6.04 (s, 2H, 2-NH$_2$, exch), 6.75–6.77 (m, 1H, Ar), 6.93–6.97 (m, 3H, Ar and 4-NH$_2$), 7.11–7.24 (m, 3H, Ar), 7.29–7.32 (m, 1H, Ar), 7.39–7.41 (m, 1H, Ar), 11.49 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{17}$H$_{15}$N$_5$S·0.28CH$_3$OH: C, 68.67; H, 6.44; N, 14.13; S, 9.05. Found: C, 68.61; H, 6.45; N, 13.79; S, 9.05.

5-(*o*-Tolylthio)-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine (4a).

Using the general procedure described above, the reaction of 195 (50 mg, 0.175 mmol) with *o*-toluene thiol 198 (0.09 mL, 0.7 mmol), copper iodide (137.5 mg, 0.7 mmol) and potassium carbonate (198.7 mg, 1.4 mmol) for 5.5 hours afforded 18 mg (31%) of 4a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp decomposes at 284.7–285.8 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.39$ (s, 3H, 2'-Me), 6.08 (s, 2H, 2-NH$_2$, exch), 6.43–6.45 (d, 1H, Ar), 6.95–7.07 (m, 3H, Ar), 7.23–7.25 (m, 4H, Ar and 4-NH$_2$), 7.41–7.43 (m, 1H, Ar), 11.53 (s, 1H, 9-NH, exch). Elemental
analysis calculated (%) for C_{17}H_{15}N_{5}S·0.34CH_{3}OH: C, 62.67; H, 4.96; N, 21.08; S, 9.65.
Found: C, 62.35; H, 4.69; N, 21.23; S, 9.88.

5-(3,4-Dimethylphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (5a).
Using the general procedure described above, the reaction of 195 (40 mg, 0.14 mmol) with 3,4-dimethylphenylthiol 199 (0.07 mL, 0.56 mmol), copper iodide (110 mg, 0.56 mmol) and potassium carbonate (159 mg, 1.12 mmol) for 4.5 hours afforded 21 mg (41%) of 5a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp decomposes at 252.8–253.8 °C. $^1$H NMR (400 MHz, DMSO-$_d6$): $\delta =$ 2.10–2.11 (d, 6H, 3'-Me and 4'-Me), 6.03 (s, 2H, 2-NH$_2$, exch), 6.73–6.75 (d, 1H, Ar), 6.94–7.02 (m, 3H, Ar and 4-NH$_2$), 7.17–7.38 (m, 4H, Ar), 11.46 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{18}$H$_{17}$N$_5$S·0.58CH$_3$OH: C, 63.04; H, 5.50; N, 19.78; S, 9.06. Found: C, 63.07; H, 5.17; N, 19.61; S, 9.00.

5-(3-Methoxyphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (6a).
Using the general procedure described above, the reaction of 195 (40 mg, 0.14 mmol) with 3-methoxyphenylthiol 200 (0.07 mL, 0.56 mmol), copper iodide (110 mg, 0.56 mmol) and potassium carbonate (159 mg, 1.12 mmol) for 4.5 hours afforded 20 mg (21%) of 6a as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp decomposes at 190.4–191.3 °C. $^1$H NMR (400 MHz, DMSO-$_d6$): $\delta =$ 3.64 (s, 3H, OCH$_3$), 6.14 (bs, 2H, 2-NH$_2$, exch), 6.53–6.56 (d, 2H, Ar), 6.71–6.74 (d, 1H, Ar), 7.15–7.44 (m, 6H, Ar and 4-NH$_2$), 11.55 (s, 1H, 9-NH, exch). HRMS (ESI): $m/z$ calculated for C$_{17}$H$_{15}$N$_5$OS + H$^+$ [M+H$^+$]: 338.1076. Found: 338.1078. HPLC analysis:
retention time = 41.89 min; peak area, 97.46%; eluent A, H₂O; eluent B, ACN; gradient elution (100% H₂O to 10% H₂O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt.

5-(α-Methoxyphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (7a).

Compound 195 (50 mg, 0.18 mmol), 2-methoxyphenyl thiol 201 (76 mg, 0.54 mmol), copper bromide (16 mg, 0.14 mmol), copper oxide (16 mg, 0.14 mmol) and potassium carbonate (75 mg, 0.72 mmol) were added to a Biotage® microwave vial. DMF (3 mL) was added as solvent and the vial was sealed. The reaction was run in a Biotage® initiator at 145 °C for 9 h. After cooling to room temperature, silica gel (0.3 g) was added and DMF was removed under reduced pressure. The crude product was purified by column chromatography, sequentially eluting with 1%, 2% and 3% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford 20 mg (31%) of 7a as a brown solid. TLC $R_f = 0.65$ (CHCl₃/MeOH, 5:1 with 2 drops of conc. NH₄OH); mp >250 °C. $^1$H NMR (400 MHz, DMSO-d₆): $\delta = 3.34$ (s, 3H, 4-NH₂, exch); 3.89 (s, 3H, OCH₃); 6.14 (br, 2H, 2-NH₂, exch); 6.31–6.33 (d, 1H, Ar); 6.70–6.74 (t, 1H, Ar); 7.03–7.05 (m, 1H, Ar); 7.10–7.13 (m, 1H, Ar); 7.23–7.31 (m, 2H, Ar); 7.42–7.44 (d, 1H, Ar); 11.57 (br, 1H, 9-NH, exch). HRMS (ESI): $m/z$ calculated for C₁₇H₁₆N₅O₃ + H⁺ [M+H⁺]: 338.1070. Found: 338.1064. HPLC analysis: retention time = 31.33 min; peak area, 95.86%; eluent A, H₂O; eluent B, ACN; gradient elution (100% H₂O to 10% H₂O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt. Elemental analysis calculated (%) for C₁₇H₁₅N₅O₃·0.225CHCl₃: C, 56.80; H, 4.21; N, 19.23; S, 8.80. Found: C, 56.92; H, 4.52; N, 18.94; S, 8.62.
2-Amino-4-methylfuran-3-carbonitrile (203).

To a solution of malononitrile (1.46 g, 22.1 mmol) in 20 mL anhydrous MeOH was added triethylamine (2.23 g, 22.1 mmol) under argon atmosphere. To this solution at 0 °C was added a solution of hydroxyacetone 202 (1.64 g, 1.08 g/mL, 22.1 mmol) in 10 mL anhydrous MeOH. The solution was warmed to rt and stirred for 14 h. The solvents were evaporated to give 203 as a brown-colored crude solid. TLC $R_f = 0.60$ (hexane/EtOAc, 10:1). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 2.01$ (s, 3H, 4-CH$_3$), 4.75 (br, 2H, 2-NH$_2$), 6.61 (s, 1H, C5-CH). $^1$H NMR agreed well with the literature reported$^{143}$ values. This material was used directly for the next step without purification.

5-Methylfuro[2,3-$d$]pyrimidin-4-amine (204).

In a 500 mL round bottom flask, sodium metal (2.3 g, 100 mmol) was added cautiously to stirred anhydrous ethanol (5.8 mL, 100 mmol) over 10 min at room temperature. After stirring for another 5 min, formamidine hydrochloride (8.05 g, 100 mmol) was added. The resulting slurry was stirred at room temperature for 30 min after which solution of 203 (13 g crude, $\approx$ 100 mmol) in 200 mL anhydrous ethanol was added. The mixture was heated to reflux for 8 h. After cooling the reaction mixture to room temperature, silica gel (25 g) was added and the solvents were evaporated under reduced pressure to obtain a plug. Purification was done by flash chromatography using 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to provide 7.1 g (47%, two steps) of 204 as lustrous pink crystals. TLC $R_f = 0.29$ (CHCl$_3$/MeOH, 10:1); mp 241.9–242.2 °C (lit.$^{174}$ 240.2–242.5 °C). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.288–2.292$ (d, 3H, CH$_3$, $J = 1.4$ Hz), 7.02 (br, 2H, NH$_2$, exch), 7.533–5.536 (d, 1H, C6-...
CH, $J = 1.4$ Hz), 8.13 (s, 1H, C2-CH). $^1$H NMR agreed well with the literature reported$^{174}$ values.

**N-(4-Methoxyphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (181).**

A 50 mL round bottom flask was charged with copper iodide (66.5 mg, 0.35 mmol), anhydrous potassium carbonate (480 mg, 3.5 mmol), L-proline (80 mg, 0.7 mmol), compound 204 (150 mg, 1 mmol) and 4-iodo anisole 205 (350 mg, 3.5 mmol). The flask was connected to vacuum for 3 min followed by addition of anhydrous DMF (15 mL) using syringe. The flask was purged with argon for 5 min and then heated in an oil bath maintained at 110 °C. On heating, the color of the suspension turned blueish grey which lasted for about 2 h. The reaction was stirred for another 22 h at 110 °C at the end of which the mixture was allowed to cool to room temperature. Ethyl acetate (25 mL) was added and the mixture was poured into water (100 mL). The product was extracted with ethyl acetate (100 mL × 2). The combined organic extracts were washed with brine (100 mL) and dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (500 mg) was added and the solvent evaporated to obtain a plug. Purification by flash chromatography using hexanes and ethyl acetate (10:1 to 2:1) afforded 87.5 mg (33%) of 181 as a light brown solid. TLC $R_f = 0.77$ (CHCl$_3$/MeOH, 10:1); mp 101.0–101.7 °C (lit.$^{174}$ 99.0–101.6 °C). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.383–2.386$ (d, 3H, CH$_3$, $J = 1.2$ Hz), 3.75 (s, 3H, OCH$_3$), 6.92–6.94 (d, 2H, Ar, $J = 8.8$ Hz), 7.47–7.49 (d, 2H, Ar, $J = 8.8$ Hz), 7.650–7.653 (d, 1H, C6-CH, $J = 1.2$ Hz), 8.23 (s, 1H, C2-CH), 8.38 (s, 1H, 4-NH, exch). $^1$H NMR agreed well with the literature reported$^{174}$ values.
General procedure for the synthesis of substituted furo[2,3-d]pyrimidines 1b–4b.

In a 25 mL round bottom flask, compound 181 (1 equivalent) and DMF (2 mL) were added to afford a solution. The flask was purged with argon for 5 min followed by cooling down to 0 °C using ice bath. To the solution at 0 °C was added sodium hydride (3 equivalents) and stirred for 20 min under argon atmosphere. Appropriate alkyl iodide (3-4 equivalents) was injected to the reaction mixture and the flask was warmed to room temperature. The mixture was stirred at room temperature until the TLC showed completion of the reactant 181. Aqueous 1N HCl solution was added dropwise to quench the reaction followed by water (20 mL) to afford a precipitate. The product was extracted with ethyl acetate (10 mL × 3). The combined organic extracts were washed with brine (10 mL), dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (200 mg) was added and the solvent was evaporated to obtain a silica gel plug. The silica gel plug was purified by flash chromatography using hexanes and ethyl acetate (5:1). Fractions containing the product (TLC) were pooled and concentrated to obtain 1b–4b in yields of 57–85%.

\( N\)-Ethyl-\( N\)-(4-methoxyphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (1b).

Using the general procedure described above, ethyl iodide 206 (61.70 mg or 0.1 mL, 1.2 mmol) was added to an ice-cold solution of 181 (100 mg, 0.4 mmol) and NaH (28.8 mg, 1.2 mmol) in 2 mL DMF and the reaction was run at room temperature for 2 h, to provide 94 mg (85%) of 1b as an off-white solid. TLC \( R_f = 0.88 \) (CHCl₃/MeOH, 15:1); mp 77.8–78.3 °C. \( ^1\)H NMR (400 MHz, CDCl₃): \( \delta = 1.064–1.067 \) (d, 3H, 5-CH₃, \( J = 1.2 \) Hz), 1.22 (t, 3H, CH₃, \( J = 7 \) Hz), 3.82 (s, 3H, OCH₃), 4.06–4.11 (q, 2H, NCH₂, \( J = 7 \) Hz),
6.88–6.91 (d, 2H, Ar, J = 8.9 Hz), 7.074–7.077 (d, 1H, C6-CH, J = 1.2 Hz), 7.09–7.11 (d, 2H, Ar, J = 8.9 Hz), 8.48 (s, 1H, C2-CH). Elemental analysis calculated (%) for C16H11N3O2: C, 67.83; H, 6.05; N, 14.83. Found: C, 67.53; H, 6.05; N, 14.77.

*N*-Isopropyl-*N*-(4-methoxyphenyl)-5-methylfuro[2,3-\textit{d}]*pyrimidin-4-amine (2b).

Using the general procedure described above, isopropyl iodide 207 (266.36 mg, 1.6 mmol) was added to an ice-cold solution of 181 (100 mg, 0.4 mmol) and NaH (38.4 mg, 1.6 mmol) in 2 mL DMF and the reaction was run at room temperature for 6 h, to provide 75 mg (63%) of 2b as an off-white solid. TLC R\textsubscript{f} = 0.88 (CHCl\textsubscript{3}/MeOH, 15:1); mp 95.8–96.6 °C. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta = 1.02\) (d, 3H, 5-CH\textsubscript{3}, J = 1.3 Hz), 1.14–1.16 (d, 6H, two CH\textsubscript{3}, J = 6.8 Hz), 3.79 (s, 3H, OCH\textsubscript{3}), 5.30–5.37 (m, 1H, N-CH), 6.85–6.87 (d, 2H, Ar, J = 8.9 Hz), 7.000–7.004 (d, 1H, C6-CH, J = 1.3 Hz), 7.02–7.04 (d, 2H, Ar, J = 8.9 Hz), 8.42 (s, 1H, C2-CH). Elemental analysis calculated (%) for C\textsubscript{17}H\textsubscript{19}N\textsubscript{3}O\textsubscript{2}: C, 68.67; H, 6.44; N, 14.13. Found: C, 68.61; H, 6.45; N, 13.79.

*N*-(4-Methoxyphenyl)-5-methyl-*N*-propylfuro[2,3-\textit{d}]*pyrimidin-4-amine (3b).

Using the general procedure described above, propyl iodide 208 (199 mg or 0.12 mL, 1.2 mmol) was added to an ice-cold solution of 181 (100 mg, 0.4 mmol) and NaH (28.8 mg, 1.2 mmol) in 2 mL DMF and the reaction was run at room temperature for 2 h, to provide 80 mg (69%) of 3b as an off-white solid. TLC R\textsubscript{f} = 0.88 (CHCl\textsubscript{3}/MeOH, 15:1); mp 113.5–114.2 °C. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta = 0.94–0.97\) (t, 3H, CH\textsubscript{3}), 1.095–1.098 (d, 3H, 5-CH\textsubscript{3}, J = 1.2 Hz), 1.70–1.74 (m, 2H, CH\textsubscript{2}), 3.84 (s, 3H, OCH\textsubscript{3}), 3.96–3.99 (m, 2H, N-CH\textsubscript{2}), 6.89–6.91 (d, 2H, Ar, J = 8.95 Hz), 7.092–7.095 (d, 1H, C6-CH, J = 1.2
Hz), 7.10–7.12 (d, 2H, Ar, \( J = 9.0 \) Hz), 8.49 (s, 1H, C2-CH). Elemental analysis calculated (%) for \( \text{C}_{17}\text{H}_{10}\text{N}_{3}\text{O}_{2} \): C, 68.67; H, 6.44; N, 14.13. Found: C, 68.72; H, 6.41; N, 14.16.

**N-Butyl-N-(4-methoxyphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (4b).**

Using the general procedure described above, butyl iodide 209 (216.26 mg or 0.13 mL, 1.2 mmol) was added to an ice-cold solution of 181 (100 mg, 0.4 mmol) and NaH (28.8 mg, 1.2 mmol) in 2 mL DMF and the reaction was run at room temperature for 2.5 h to provide 70 mg (57%) of 4b as a semisolid. TLC \( R_f = 0.88 \) (CHCl3/MeOH, 15:1); \(^1\)H NMR (400 MHz, CDCl3): \( \delta = 0.88–0.92 \) (t, 3H, CH3), 1.06 (s, 3H, 5-CH3), 1.30–1.39 (m, 2H, CH2), 1.59–1.67 (m, 2H, CH2), 3.81 (s, 3H, OCH3), 3.96–4.00 (m, 2H, N-CH2), 6.86–6.88 (d, 2H, Ar, \( J = 8.9 \) Hz), 7.06–7.09 (m, 3H, Ar and C6-CH), 8.45 (s, 1H, C2-CH). HRMS (ESI): \( m/z \) calculated for \( \text{C}_{18}\text{H}_{21}\text{N}_{3}\text{O}_{2} + \text{H}^+ \) [M+H\(^+\)]: 312.1712. Found: 312.1709. HPLC analysis: retention time = 38.76 min; peak area, 97.33%; eluent A, H2O; eluent B, ACN; gradient elution (100% H2O to 10% H2O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt.

**General procedure for the synthesis of N-(4-substituted phenyl)-5-methylfuro[2,3-d]pyrimidin-4-amines 213–215 using L-proline as the ligand.**

A 50 mL round bottom flask was charged with copper iodide (66.5 mg, 0.35 mmol), anhydrous potassium carbonate (480 mg, 3.5 mmol), L-proline (80 mg, 0.7 mmol), compound 204 (150 mg, 1 mmol) and appropriate iodobenzene (3.5 mmol). The flask was connected to vacuum for 3 min followed by addition of anhydrous DMF (15 mL)
using syringe. The flask was purged with argon for 5 min and then heated in an oil bath maintained at 110 °C. On heating, the color of the suspension turned blueish grey which lasted for about 2 h. The reaction was stirred for additional 22 h at 110 °C at the end of which the mixture was allowed to cool to room temperature. Ethyl acetate (25 mL) was added and the mixture was poured into water (100 mL). The product was extracted with ethyl acetate (100 mL × 2). The combined organic extracts were washed with brine (100 mL) and dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (500 mg) was added and the solvent evaporated to obtain a plug. Purification was performed by flash chromatography using hexanes and ethyl acetate (10:1 to 2:1). Fractions containing the product (TLC) were pooled and evaporated to afford the product.

*N-(4-Ethylphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (213).*

Using the general procedure described above, reaction between 204 (150 mg, 1 mmol) and 1-ethyl-4-iodobenzene 210 (350 mg, 3.5 mmol) provided 74 mg (29%) of 213 as a brown semisolid. TLC $R_f = 0.78$ (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 1.23$–1.27 (t, 3H, CH$_3$), 2.421–2.423 (d, 3H, 5-CH$_3$, $J = 0.9$ Hz), 2.65–2.69 (q, 2H, CH$_2$), 6.85 (s, 1H, 4-NH, exch), 7.21–7.23 (d, 2H, Ar, $J = 8.3$ Hz), 7.296–7.298 (d, 1H, C6-CH, $J = 0.9$ Hz), 7.51–7.53 (d, 2H, Ar, $J = 8.3$ Hz), 8.45 (s, 1H, C2-CH). Elemental analysis calculated (%) for C$_{15}$H$_{15}$N$_3$O·0.1CH$_3$CO$_2$C$_2$H$_5$: C, 70.57; H, 6.08; N, 16.03. Found: C, 70.79; H, 6.20; N, 15.75.
5-Methyl-N-(4-(methylthio)phenyl)furo[2,3-d]pyrimidin-4-amine (214).

Using the general procedure described above, reaction between 204 (150 mg, 1 mmol) and 4-iodo-thioanisole 211 (350 mg, 3.5 mmol) afforded 40 mg (15%) of 214 as a semisolid. TLC $R_f = 0.83$ (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 2.41$ (s, 3H, SCH$_3$), 2.51 (s, 3H, 5-CH$_3$), 7.02 (s, 1H, 4-NH, exch), 7.28–7.34 (m, 3H, Ar and C6-CH), 7.57–7.59 (d, 2H, Ar), 8.47 (s, 1H, C2-CH).

$N$-(4-Nitrophenoxy)-5-methylfuro[2,3-d]pyrimidin-4-amine (215).

Using the general procedure described above, reaction between 204 (150 mg, 1 mmol) and 4-iodo-nitrobenzene 212 (250 mg, 3.5 mmol) provided 51 mg (19%) of 215 as a brown solid. TLC $R_f = 0.79$ (CHCl$_3$/MeOH, 10:1); $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 2.474$–2.476 (d, 3H, CH$_3$), 7.835–7.838 (d, 1H, C6-CH), 7.98–8.00 (d, 2H, Ar), 8.25–8.27 (d, 2H, Ar), 8.54 (s, 1H, C2-CH), 9.16 (s, 1H, 4-NH, exch). HRMS (ESI): $m/z$ calculated for C$_{13}$H$_{11}$N$_4$O$_3$ + H$^+$ [M+H$^+$]: 271.0831. Found: 271.0836.

$N$-Methyl-$N$-(4-ethylphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (1c).

In a 25 mL round bottom flask, compound 213 (40 mg, 0.16 mmol, 1 equivalent) and DMF (2 mL) were added to afford a solution. The flask was purged with argon for 5 min followed by cooling down to 0 °C using ice bath. To the solution at 0 °C was added sodium hydride (11.5 mg, 0.48 mmol) and stirred for 20 min. Dimethyl sulfate (0.04 mL, 0.48 mmol) was injected to the reaction mixture and the flask was warmed to room temperature. The mixture was stirred at room temperature for 16 h and the TLC showed complete utilization of the reactant 213. Aqueous 1N HCl solution was added dropwise to
quench the reaction followed by water (20 mL) to afford a precipitate. The product was extracted with ethyl acetate (10 mL × 3). The combined organic extracts were washed with brine (10 mL), dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (200 mg) was added and the solvent was evaporated to obtain a plug which was purified by flash chromatography using hexanes and ethyl acetate (10:1) to obtain 23 mg (53%) of 1c as a white solid. TLC $R_f = 0.77$ (CHCl₃/MeOH, 10:1); mp 116.3–116.8 °C. $^1$H NMR (400 MHz, CDCl₃): $\delta = 1.09$ (s, 3H, 5-CH₃), 1.24–1.28 (t, 3H, CH₃), 2.64–2.70 (q, 2H, CH₂), 3.57 (s, 3H, NCH₃), 7.10–7.12 (d, 3H, Ar), 7.19–7.21 (m, 2H, Ar and C6-CH), 8.56 (s, 1H, C2-CH). Elemental analysis calculated (%) for C₁₆H₁₇N₃O·0.07CH₃CO₂C₂H₅: C, 71.50; H, 6.47; N, 15.36. Found: C, 71.29; H, 6.28; N, 15.55.

**General procedure for the synthesis of N-(4-substitutedphenyl)-5-methylfuro[2,3-d]pyrimidin-4-amines 214, 215, 218 and 219 using pipecolinic acid as the ligand.**

A 50 mL round bottom flask was charged with compound 204 (1 equivalent), copper iodide (0.4–0.5 equivalents), anhydrous potassium carbonate (2–4 equivalents), pipecolinic acid (0.8–1.0 equivalents) and appropriate iodobenzene (2–3 equivalents). The flask was connected to vacuum for 3 min followed by addition of anhydrous DMF using syringe. The flask was purged with argon for 5 min and then heated in an oil bath maintained at 110 °C. On heating, the color of the suspension turned orange/pink suspension which lasted for about 2 h. The reaction was stirred for additional 22 h at 110 °C at the end of which the mixture was allowed to cool to room temperature. Ethyl acetate (25 mL) was added and the mixture was poured into water (100 mL). The product
was extracted with ethyl acetate (100 mL × 2). The combined organic extracts were washed with brine (100 mL) and dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (500 mg) was added and the solvent evaporated to obtain a plug. Purification was performed by flash chromatography using hexanes and ethyl acetate (10:1 to 2:1). Fractions containing the product (TLC) were pooled and evaporated to afford the product.

5-Methyl-N-(4-(methylthio)phenyl)furo[2,3-d]pyrimidin-4-amine (214). Using the general procedure described above, reaction between 204 (149 mg, 1 mmol) and 4-iodo-thioanisole 211 (500 mg, 2 mmol) in the presence of copper iodide (76 mg, 0.4 mmol), pipecolinic acid (103 mg, 0.8 mmol), anhydrous potassium carbonate (277 mg, 2 mmol) and anhydrous DMF (15 mL) afforded 120 mg (43%) of 214 as a brownish red solid. TLC $R_f = 0.83$ (CHCl$_3$/MeOH, 10:1); mp 112.8–113.0 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.431–2.434$ (d, 3H, 5-CH$_3$, $J = 1.2$ Hz), 2.49 (s, 3H, SCH$_3$), 7.28–7.30 (d, 2H, Ar, $J = 8.6$ Hz), 7.62–7.63 (d, 2H, Ar, $J = 8.6$ Hz), 7.705–7.708 (d, 1H, C$_6$-CH, $J = 1.2$ Hz), 8.33 (s, 1H, C2-CH), 8.48 (s, 1H, 4-NH, exch). Elemental analysis calculated (%) for C$_{14}$H$_{13}$N$_3$OS·0.07CH$_3$(CH$_2$)$_4$CH$_3$: C, 62.44; H, 5.08; N, 15.15; S, 11.77. Found: C, 62.41; H, 5.13; N, 15.02; S, 11.74.

N-(4-Nitrophenyl)-5-methylfuro[2,3-d]pyrimidin-4-amine (215). Using the general procedure described above, reaction between 204 (149 mg, 1 mmol) and 4-iodo-nitrobenzene 212 (500 mg, 2 mmol) in the presence of copper iodide (76 mg, 0.4 mmol), pipecolinic acid (103 mg, 0.8 mmol), anhydrous potassium carbonate (277
mg, 2 mmol) and anhydrous DMF (15 mL) afforded 90 mg (33%) of 215 as yellow crystals. TLC $R_f = 0.79$ (CHCl₃/MeOH, 10:1); mp decomposes at 157.6–158.2 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.472–2.474$ (d, 3H, 5-CH₃, $J = 1.0$ Hz), 7.832–7.835 (d, 1H, C6-CH, $J = 1.2$ Hz), 7.98–8.00 (d, 2H, Ar, $J = 9.2$ Hz), 8.25–8.27 (d, 2H, Ar, $J = 9.2$ Hz), 8.54 (s, 1H, C2-CH), 9.14 (s, 1H, 4-NH, exch). Elemental analysis calculated (%) for C13H10N4O3·0.09CH3(CH2)4CH3: C, 58.50; H, 4.08; N, 20.15. Found: C, 58.18; H, 4.17; N, 19.82.

$N^1$-Methyl-$N^4$-(5-methylfuro[2,3-$d$]pyrimidin-4-yl)benzene-1,4-diamine (218).

Using the general procedure described above, reaction between 204 (298 mg, 2 mmol) and 4-iodo-$N$-methyl aniline 216 (700 mg, 6 mmol) in the presence of copper iodide (190 mg, 1 mmol), pipercolinic acid (258 mg, 2 mmol), anhydrous potassium carbonate (1100 mg, 8 mmol) and anhydrous DMF (30 mL) provided 136 mg (27%) of 218 as a light brown solid. TLC $R_f = 0.73$ (CHCl₃/MeOH, 10:1); mp 161.4–162.2 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.355–2.357$ (d, 3H, 5-CH₃, $J = 1.0$ Hz), 2.68–2.69 (d, 3H, 4'-NCH₃, $J = 5.0$ Hz), 5.56–5.60 (q, 1H, 4'-NH, exch), 6.53–6.56 (d, 2H, Ar, $J = 8.7$ Hz), 7.23–7.26 (d, 2H, Ar, $J = 8.7$ Hz), 7.618–7.621 (d, 1H, C6-CH, $J = 1.2$ Hz), 8.19 (s, 1H, C2-CH), 8.26 (s, 1H, 4-NH, exch). Elemental analysis calculated (%) for C14H14N4O: C, 66.13; H, 5.55; N, 22.03. Found: C, 66.14; H, 5.57; N, 21.90.

$N^1,N^1$-Dimethyl-$N^4$-(5-methylfuro[2,3-$d$]pyrimidin-4-yl)benzene-1,4-diamine (219).

Using the general procedure described above, reaction between 204 (298 mg, 2 mmol) and 4-iodo-$N,N$-dimethyl aniline 217 (741 mg, 6 mmol) in the presence of copper iodide
(190 mg, 1 mmol), pipercolinic acid (258 mg, 2 mmol), anhydrous potassium carbonate (1100 mg, 8 mmol) and anhydrous DMF (30 mL) provided 191 mg (36%) of 219 as a brown solid. TLC $R_f = 0.77$ (CHCl$_3$/MeOH, 10:1); mp decomposes at 196.8–197.2 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.379–2.382$ (d, 3H, 5-CH$_3$, $J = 1.1$ Hz), 2.89 (s, 6H, 4'-N(CH$_3$_2)), 6.74–6.76 (d, 2H, Ar, $J = 8.98$ Hz), 7.36–7.38 (d, 2H, Ar, $J = 8.93$ Hz), 7.637–7.640 (d, 1H, C6-CH, $J = 1.2$ Hz), 8.21 (s, 1H, C2-CH), 8.30 (s, 1H, 4-NH, exch). Elemental analysis calculated (%) for C$_{15}$H$_{16}$N$_4$O·0.1CH$_3$(CH$_2$)$_4$CH$_3$: C, 67.66; H, 6.33; N, 20.23. Found: C, 67.48; H, 6.14; N, 19.92.

**General procedure for the synthesis of substituted furo[2,3-d]pyrimidines 2c, 4c and 5c.**

In a 25 mL round bottom flask, appropriate furo[2,3-d]pyrimidine (1 equivalent) and DMF (2 mL) were added to afford a solution. The flask was purged with argon for 5 min followed by cooling down to 0 °C using ice bath. To the solution at 0 °C was added sodium hydride (3 equivalents) and stirred for 20 min under argon atmosphere. Dimethyl sulfate (3 equivalents) was injected to the reaction mixture and the flask was warmed to room temperature. The mixture was stirred at room temperature for 4 h after which aqueous 1N HCl solution was added dropwise to quench the reaction followed by water (20 mL) to afford a precipitate. The product was extracted with ethyl acetate (10 mL × 3) and the combined organic extracts were washed with brine (10 mL), dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (200 mg) was added and the solvent was evaporated to obtain a plug. The plug was purified by flash
chromatography eluting with hexanes and ethyl acetate (5:1) to obtain 2c, 4c and 5c in yields of 43-51%.

\( \text{N,5-Dimethyl-N-(4-(methylthio)phenyl)furo[2,3-d]pyrimidin-4-amine (2c).} \)

Using the general procedure described above, reaction between 212 (105 mg, 0.4 mmol) and dimethylsulfate (0.08 mL, 1.2 mmol) in 8 mL DMF in the presence of sodium hydride (28.8 mg, 1.2 mmol) provided 56 mg (51%) of 2c as a brown solid. TLC \( R_f = 0.88 \) (CHCl\(_3\)/MeOH, 10:1); mp 95.8–96.2 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \( \delta = 1.109–1.112 \) (d, 3H, 5-CH\(_3\), \( J = 1.1 \) Hz), 2.48 (s, 3H, SCH\(_3\)), 3.47 (s, 3H, NCH\(_3\)), 7.20–7.22 (d, 2H, Ar, \( J = 8.65 \) Hz), 7.28–7.30 (d, 2H, Ar, \( J = 8.66 \) Hz), 7.567–7.570 (d, 1H, C6-CH, \( J = 1.2 \) Hz), 8.50 (s, 1H, C2-CH). Elemental analysis calculated (% for C\(_{15}\)H\(_{15}\)N\(_3\)O\(_S\): C, 63.13; H, 5.30; N, 14.72; S, 11.24. Found: C, 63.17; H, 5.41; N, 14.76; S, 11.14.

\( \text{N}^1,\text{N}^3,\text{N}^4-\text{Trimethyl-N}^d-(5\text{-methylfuro[2,3-d]pyrimidin-4-yl)benzene-1,4-diamine (4c).} \)

Using the general procedure described above, reaction between 219 (107.3 mg, 0.4 mmol) and dimethylsulfate (0.08 mL, 1.2 mmol) in 8 mL DMF in the presence of sodium hydride (28.8 mg, 1.2 mmol) provided 54 mg (45%) of 4c as a white solid. TLC \( R_f = 0.85 \) (CHCl\(_3\)/MeOH, 10:1); mp 142.1–142.8 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \( \delta = 1.071–1.073 \) (d, 3H, 5-CH\(_3\), \( J = 0.9 \) Hz), 2.90 (s, 6H, 4'-N(CH\(_3\))\(_2\)), 3.41 (s, 3H, 4-NCH\(_3\)), 6.73–6.75 (d, 2H, Ar, \( J = 8.9 \) Hz), 7.07–7.09 (d, 2H, Ar, \( J = 8.9 \) Hz), 7.482–7.484 (d, 1H, C6-CH, \( J = 0.9 \) Hz), 8.41 (s, 1H, C2-CH). Elemental analysis calculated (%) for
N,N-Dimethyl-N-(4-nitrophenyl)furo[2,3-d]pyrimidin-4-amine (5c).

Using the general procedure described above, reaction between 215 (48.5 mg, 0.18 mmol) and dimethylsulfate (0.04 mL, 0.54 mmol) in 4 mL DMF in the presence of sodium hydride (13 mg, 0.54 mmol) provided 22 mg (43%) of 5c as a yellow solid. TLC Rf = 0.83 (CHCl3/MeOH, 10:1); mp 190.6–191.0 °C. 1H NMR (400 MHz, DMSO-d6): δ = 1.44 (s, 3H, 5-CH3), 3.63 (s, 3H, NCH3), 7.29–7.31 (d, 2H, Ar, J = 9.03 Hz), 7.82 (s, 1H, C6-CH), 8.16–8.18 (d, 2H, Ar, J = 9.07 Hz), 8.74 (s, 1H, C2-CH). 1H NMR (400 MHz, CDCl3): δ = 1.442–1.444 (d, 3H, 5-CH3, J = 0.9 Hz), 3.67 (s, 3H, NCH3), 7.08–7.11 (d, 2H, Ar, J = 9.1 Hz), 7.305–7.307 (d, 1H, C6-CH, J = 0.9 Hz), 8.14–8.16 (d, 2H, Ar, J = 9.1 Hz), 8.69 (s, 1H, C2-CH). Elemental analysis calculated (%) for C14H12N4O3·0.33CH3CO2C2H5: C, 58.72; H, 4.71; N, 17.88. Found: C, 59.04; H, 4.72; N, 17.82.

N1,N4-Dimethyl-N1-(5-methylfuro[2,3-d]pyrimidin-4-yl)benzene-1,4-diamine (3c).

In a 25 mL round bottom flask, compound 218 (50.8 mg, 0.2 mmol, 1 equivalent) and DMF (3 mL) were added to afford a solution. The flask was purged with argon for 5 min followed by cooling down to 0 °C using ice bath. To the solution at 0 °C was added sodium hydride (5.3 mg, 0.22 mmol) and stirred for 20 min at 0 °C. Dimethyl sulfate (0.02 mL, 0.22 mmol) was injected to the reaction mixture and the mixture was stirred at -10–0 °C for 48 h after which TLC showed complete utilization of the reactant 218.
Aqueous 1N HCl solution was added dropwise to quench the reaction followed by water (5 mL) to afford a precipitate. The product was extracted with ethyl acetate (10 mL × 3). The combined organic extracts were washed with brine (10 mL), dried (anhydrous sodium sulfate), added silica gel (200 mg) and concentrated under reduced pressure to obtain a plug. Purification was done by flash chromatography eluting with hexanes and ethyl acetate (10:1) to obtain 12.5 mg (20%) of 3c as a brown solid and 18 mg (30%) of undesired product 4c. TLC $R_f = 0.82$ (CHCl3/MeOH, 10:1); mp 122.1–122.9 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.103–1.105$ (d, 3H, 5-CH$_3$, $J = 1.1$ Hz), 2.67–2.68 (d, 3H, 4'-NCH$_3$, $J = 5.0$ Hz), 3.39 (s, 3H, 4-NCH$_3$), 5.80–5.84 (q, 1H, 4'-NH, exch), 6.54–6.56 (d, 2H, Ar, $J = 8.7$ Hz), 6.98–7.00 (d, 2H, Ar, $J = 8.7$ Hz), 7.474–7.477 (d, 1H, C6-CH, $J = 1.2$ Hz), 8.39 (s, 1H, C2-CH). Elemental analysis calculated (%) for C$_{15}$H$_{16}$N$_4$O·0.6C$_4$H$_8$O: C, 67.07; H, 6.73; N, 17.98. Found: C, 67.32; H, 6.58; N, 17.91.

**Ethyl(2-chloro-2-nitrophenyl)(cyano)acetate (8).**

To an ice cold solution of ethyl cyanoacetate (10.9 mL, 102.4 mmol) in anhydrous THF (170 mL) under nitrogen, was added potassium tert–butoxide (12.7 g, 107.5 mmol). The formed white suspension was stirred for 15 min, then treated with 1,2–dichloro-3-nitrobenzene 7 (9.83 g , 51.2 mmol). The suspension was heated to reflux for 48 hours. The resulting reddish brown solution was poured in to 50 mL water, and the aqueous mixture was then acidified to pH 2 with conc. HCl. The mixture was extracted with ether (150 mL × 3) and then the combined organic phase was dried using anhydrous Na$_2$SO$_4$ and then concentrated to give a dark yellow–colored semisolid. Column chromatography in a column packed with silica gel, four times the weight of the dark oil using 10:1
hexane:ethyl acetate as the eluent provided 13.2 g (96%) of 8 as a viscous yellow liquid. TLC $R_f = 0.32$ (hexane/EtOAc, 3:1). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.18–1.21$ (t, 3H, CH$_3$), 4.19–4.24 (q, 2H, CH$_2$), 6.37 (s, 1H, CH), 7.7–8.15 (m, 3H, Ar). $^1$H NMR and mp agreed well with the literature reported$^{125}$ values.

**Ethyl-2-amino-4-chloro-1H-indole-3-carboxylate (9).**

To a solution of 8 (6 g, 22.34 mmol) in 230 mL glacial acetic acid was added 6 g of zinc dust. The mixture was heated to 55 °C for 1 h and then 2 g of Zn dust was added. Zinc dust (2 g) was added for every 10 hours. After stirring at 55 °C for another 45 hrs, the yellow mixture was filtered through a pad of Celite. The pad was washed with acetic acid and the filtrate was concentrated to a residue that was distributed between chloroform and water. The organic phase was washed with 5% NaHCO$_3$ solution to provide a pink precipitate which was filtered, dried over P$_2$O$_5$, dissolved in acetone, added silica gel (equal to the quantity of the precipitate) and converted to a silica gel plug by removing the solvent under reduced pressure. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with 1% methanol in CHCl$_3$. Fractions containing the product (TLC) were pooled and evaporated to give 3.94 g (74%) of 9 as a pink solid. TLC $R_f = 0.40$ (CHCl$_3$/MeOH, 10:1); mp 141.4–142.0 °C (lit.$^{125}$ 140.0–142.0 °C). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.26–1.29$ (t, 3H, CH$_3$), 4.16–4.21 (q, 2H, CH$_2$), 6.87 (s, 2H, 2-NH$_2$, exch), 6.83–7.08 (m, 3H, Ar), 10.93 (s, 1H, 9-NH, exch). $^1$H NMR agreed well with the literature reported$^{125}$ values.
2-Amino-5-chloro-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (10).
Methyl sulfone (30 g), compound 9 (4 g, 16.7 mmol) and carbamimidic chloride hydrochloride\(^\text{105}\) (2.12 g, 18.4 mmol) were taken in a round-bottomed flask and then 3 g of methyl sulfone was added on top of the mixture. Heating to 120 °C with stirring, the mixture was dissolved completely following which the reaction was continued for 40 minutes. About 100 mL water was added to quench the reaction. Neutralization of the reaction mixture with conc. NH\(_4\)OH solution resulted in a brown precipitate. The precipitate was filtered, dried under P\(_2\)O\(_5\) to provide 2.52 g (64%) of 10 as a dark brown solid. This material was used for the next step without further purification. TLC \(R_f = 0.43\) (CHCl\(_3\)/MeOH, 5:1); mp >250 °C (lit.\(^\text{125}>250\) °C). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)): \(\delta = 6.57\) (br, 2H, 2-NH\(_2\), exch), 7.04–7.17 (m, 3H, Ar), 10.41 (s, 1H, 9-NH, exch), 11.64 (s, 1H, 3-NH, exch). \(^1\)H NMR agreed well with the literature reported\(^\text{125}\) values.

\(N\)-(5-Chloro-4-oxo-4,9-dihydro-3H-pyrimido[4,5-b]indol-2-yl)-2,2-dimethyl propanamide (220).
Compound 10 (2 g, 8.5 mmol) and 150 mL of pivalic anhydride were taken in a 250 mL round bottomed flask. The reaction mixture was stirred at 120 °C for 2.5 h. Then, hexane was added under room temperature which resulted in precipitation of pale brown colored solid. The solid was filtered, washed with hexane and used without further purification for the next step. The filtrate was then concentrated and a plug was made, using silica gel three times the product. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug and eluted with 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford 1.6 g (60%)
of **220** as a brown solid. TLC $R_f = 0.80$ (CHCl₃/MeOH, 5:1); mp 189.2–189.8 °C (lit.¹²⁵ 185.8–190.1 °C). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.26$ (s, 9H, C(CH₃)₃), 7.19–7.37 (m, 3H, Ar), 11.16 (s, 1H, 2-NH, exch), 11.93 (s, 1H, 3-NH, exch), 12.11 (s, 1H, 9-NH, exch). $^1$H NMR agreed well with the literature reported¹²⁵ values.

**N-(4,5-Dichloro-9H-pyrimido[4,5-b]indol-2-yl)-2,2-dimethyl propanamide (221).**

Compound **220** (1 g, 3.137 mmol) was treated with 200 mL of POCl₃ in a 500 mL round bottom flask. The reaction mixture was heated to reflux for 4 hours. The POCl₃ was evaporated and the mixture was neutralized using conc. NH₄OH solution (28-30 wt% in water). The aqueous mixture was filtered (the precipitate being the compound) and the precipitate was dried. To the solution was added silica and solvent was removed under reduced pressure to provide a silica gel plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with chloroform and 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to provide 710 mg (67%) of **221** as a brown solid. TLC $R_f = 0.86$ (CHCl₃/MeOH, 5:1); mp 245.9–246.5 °C (lit.¹²⁵ 245.6–246.1 °C). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.24$ (s, 9H, C(CH₃)₃), 7.36–7.50 (m, 3H, Ar), 10.30 (s, 1H, 9-NH, exch), 12.96 (s, 1H, 2-NH, exch). $^1$H NMR agreed well with the literature reported¹²⁵ values.

**General procedure for the synthesis of 5-chloro-$N^4$-methyl-9H-pyrimido[4,5-b]indole-2,4-diamines 1d–6d.**

To a solution of **221** (1 equivalent) in 40 mL of i-propanol was added $N$-methyl anilines **222–226** or 6-methoxy-1,2,3,4-tetrahydroquinoline **227** and 2 drops of conc. HCl. The
reaction mixture was heated to reflux for 70 h, cooled to room temperature and then neutralized with 4 mL of 1N sodium hydroxide solution. The reaction was then heated to reflux for 2 h. The solvent was then evaporated to obtain a dark brown colored solid. The resulting precipitate was then dissolved in chloroform and methanol. To the solution was added silica gel, four times the weight of the reaction mixture, and the solvent was removed under reduced pressure to provide a plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug and was eluted with 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to give a solid which was further purified by washing with hexane.

5-Chloro-N^4^-methyl-N^4^-phenyl-9H-pyrimido[4,5-b]indole-2,4-diamine (1d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with N-methyl aniline 222 (95 mg, 0.90 mmol) to afford 64 mg (67%) of 1d as a brown solid. TLC R_f = 0.42 (CHCl_3/MeOH, 15:1); mp 228.1–229.1 °C. ^1H NMR (400 MHz, DMSO-d_6): δ = 3.34 (s, 3H, NCH_3), 6.61 (s, 2H, NH_2, exh), 6.76–6.80 (m, 3H, Ar), 7.02–7.04 (m, 1H, Ar), 7.12–7.21 (m, 3H, Ar), 7.27–7.29 (m, 1H, Ar), 11.81 (s, 1H, 9-NH, exh). Elemental analysis calculated (%) for C_{17}H_{14}ClN_{5}: C, 63.06; H, 4.36; N, 21.63; Cl, 10.95. Found: C, 63.06; H, 4.25; N, 21.62; Cl, 10.86.

5-Chloro-N^4^-methyl-N^4^-p-tolyl-9H-pyrimido[4,5-b]indole-2,4-diamine (2d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with N-methyl-p-toluidine 223 (108 mg, 0.90 mmol) to provide 60 mg (60%) of 2d as a brown solid. TLC R_f = 0.38 (CHCl_3/MeOH, 15:1); mp 231.9–232.8 °C. ^1H NMR
(400 MHz, DMSO-d$_6$): $\delta$ = 2.19 (s, 3H, CH$_3$), 3.32 (s, 3H, NCH$_3$), 6.53 (s, 2H, NH$_2$, exch), 6.70–6.72 (m, 2H, Ar), 6.94–6.96 (m, 2H, Ar), 7.00–7.02 (m, 1H, Ar), 7.16–7.19 (m, 1H, Ar), 7.25–7.27 (m, 1H, Ar), 11.74 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{18}$H$_{16}$ClN$_5$: C, 63.99; H, 4.77; N, 20.73; Cl, 10.49. Found: C, 63.85; H, 4.83; N, 20.44; Cl, 10.56.

5-Chloro-N$^4$-(4-chlorophenyl)-N$^4$-methyl-9H-pyrimido[4,5-b]indole-2,4-diamine (3d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with 4-chloro-N-methyl aniline 224 (126 mg, 0.90 mmol) to afford 68 mg (64%) of 3d as a brown solid. TLC $R_f$ = 0.39 (CHCl$_3$/MeOH, 15:1); mp 226.4–227.2 °C. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ = 3.35 (s, 3H, NCH$_3$), 6.66 (s, 2H, NH$_2$, exch), 6.75–6.77 (m, 2H, Ar), 7.04–7.06 (m, 1H, Ar), 7.16–7.19 (m, 3H, Ar), 7.28–7.30 (m, 1H, Ar), 11.86 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{17}$H$_{13}$Cl$_2$N$_5$·1.0CH$_3$OH: C, 55.40; H, 4.39; N, 17.94; Cl, 18.17. Found: C, 55.34; H, 4.33; N, 18.04; Cl, 18.06.

5-Chloro-N$^4$-3-methoxyphenyl-N$^4$-methyl-9H-pyrimido[4,5-b]indole-2,4-diamine (4d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with 3-methoxy-N-methyl aniline 225 (122 mg, 0.90 mmol) to give 65 mg (62%) of 4d as a brown solid. TLC $R_f$ = 0.39 (CHCl$_3$/MeOH, 15:1); mp 226.8–227.2 °C. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ = 3.34 (s, 3H, OCH$_3$), 3.59 (s, 3H, NCH$_3$), 6.28–6.38 (m, 3H, Ar), 6.63 (s, 2H, NH$_2$, exch), 7.00–7.05 (m, 2H, Ar), 7.18–7.22 (m, 1H, Ar);
7.28–7.30 (m, 1H, Ar); 11.82 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C_{18}H_{16}ClN_{5}O\cdot0.23CH_{3}COCH_{3}: C, 61.14; H, 4.77; N, 19.07; Cl, 9.66. Found: C, 60.88; H, 4.57; N, 19.14; Cl, 9.38.

5-Chloro-N^4-3-fluorophenyl-N^4-methyl-9H-pyrimido[4,5-b]indole-2,4-diamine (5d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with 3-fluoro-N-methyl aniline 226 (122 mg, 0.90 mmol) to yield 59 mg (58%) of 5d as a brown solid. TLC $R_f = 0.39$ (CHCl$_3$/MeOH, 15:1); mp 217.1–217.9 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 3.31$ (s, 3H, NCH$_3$), 6.49–6.56 (m, 3H, Ar), 6.71 (s, 2H, NH$_2$, exch), 7.05–7.07 (d, 1H, Ar), 7.09–7.11 (m, 1H, Ar), 7.20–7.24 (t, 1H, Ar), 7.29–7.31 (d, 1H, Ar), 11.89 (s, 1H, 9-NH, exch). HRMS (ESI): $m/z$ calculated for C$_{17}$H$_{14}$N$_5$ClF $+ \text{H}^+$ [M+H$^+$]: 342.0916. Found: 342.0911. HPLC analysis: retention time = 39.47 min; peak area, 96.40%; eluent A, H$_2$O; eluent B, ACN; gradient elution (100% H$_2$O to 10% H$_2$O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt.

5-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)-9H-pyrimido[4,5-b]indol-2-amine (6d).

Using the general procedure described above, compound 221 (100 mg, 0.30 mmol) was treated with 6-methoxy-1,2,3,4-tetrahydroquinoline 227 (145 mg, 0.90 mmol) to provide 59 mg (52%) of 6d as a brown solid. TLC $R_f = 0.30$ (CHCl$_3$/MeOH, 15:1); mp 187.1–187.9 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.78–1.79$ (m, 1H, CH), 1.98–1.99 (m, 1H, CH), 2.78–2.81 (m, 2H, CH$_2$), 3.57 (m, 1H, CH), 3.66 (s, 3H, OCH$_3$), 3.85–3.86 (m,
1H, CH), 6.42 (s, 2H, 2-NH₂, exch), 6.46–6.49 (m, 1H, Ar), 6.52–6.54 (m, 1H, Ar), 6.65–6.66 (d, 1H, Ar), 7.02–7.03 (m, 1H, Ar), 7.15–7.18 (t, 1H, Ar), 7.25–7.26 (m, 1H, Ar), 11.70 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C₂₀H₁₈ClN₅O: C, 63.24; H, 4.78; N, 18.44; Cl, 9.33. Found: C, 63.24; H, 4.97; N, 18.15; Cl, 9.31.

2-Amino-6-hydrazinopyrimidin-4(3H)-one (229).

To a stirred suspension of 228 (15 g, 103 mmol) in 250 mL water was added anhydrous hydrazine (12 g, 375 mmol), and the mixture was heated to reflux for 3 h. The resulting clear solution was cooled, and the precipitate that separated was collected by filtration, washed with water followed by ethanol and dried to give 6.6 g (46%) of 229 as a white solid. mp decomposes at 313.0 °C (lit.²⁰⁷ 314.0–315.0 °C). This material was used without further purification for the next step.

2-Amino-3,5,6,7,8,9-hexahydro-4H-pyrimido[4,5-b]indol-4-one (231).

A mixture of 2-amino-6-hydrazinopyrimidin-4(3H)-one 229 (3.5 g, 25 mmol) and cyclohexanone 230 (2.45 g, 25 mmol) in diphenyl ether (300 mL) was stirred at 120 °C for 14 h and then heated to reflux (250 °C) for 3 h. After cooling to room temperature, hexane (600 mL) was added, and the precipitated solid was collected by filtration. The solid was dried over P₂O₅, dissolved in methanol, and silica gel (three times the weight of solid) was added, following which the solvent was removed under reduced pressure to obtain a dry plug. The plug was loaded on top of a column packed with silica in chloroform. The weight of silica in the column was thirty times the weight of the plug. Flash chromatography using 20% methanol in chloroform afforded 4.1 g (81%) of 231.¹⁷⁸
as an yellow solid. TLC $R_f = 0.35$ (CHCl$_3$/MeOH, 5:1); mp >300 °C. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.64$–1.69 (m, 4H, 6-CH$_2$ and 7-CH$_2$), 2.42 (m, 2H, 5-CH$_2$), 2.53 (m, 2H, 8-CH$_2$), 5.88 (s, 2H, 2-NH$_2$, exch), 10.02 (s, 1H, 3-NH, exch), 10.50 (s, 1H, 9-NH, exch).

This material was used without further purification for the next step.

2-Amino-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (232).

A mixture of 231 (1 g, 5 mmol) and 10% palladium on carbon (480 mg, 0.5 mmol) in diphenyl ether (50 mL) was heated to reflux for 3 h. The reaction mixture was cooled to room temperature, and DMF (50 mL) was added to dissolve the product. The catalyst was removed by filtration through Celite, washed with DMF and silica gel (3 g) was added. Solvent was removed under reduced pressure to afford a plug, which was loaded on top of a silica gel column in chloroform (the silica was thirty times the weight of the plug). The column was eluted with 20% methanol in chloroform to afford 560 mg (59%) of 232$^{178}$ as an off-white solid. TLC $R_f = 0.32$ (CHCl$_3$/MeOH, 5:1); mp >300 °C. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 6.51$ (s, 2H, 2-NH$_2$), 7.03–7.10 (m, 2H, Ar), 7.22–7.25 (m, 1H, Ar), 7.68–7.70 (m, 1H, Ar), 10.47 (s, 1H, 3-NH, exch), 11.35 (s, 1H, 9-NH, exch).

This material was used without further purification for the next step.

2,2-Dimethyl-N-(4-oxo-4,9-dihydro-3H-pyrimido[4,5-b]indol-2-yl)propanamide (233).

Compound 232 (0.5 g, 2.5 mmol) and 20 mL of pivalic anhydride were taken in a 100 mL round-bottomed flask. The reaction mixture was stirred at 120 °C for 8 hours. Then, hexane (60 mL) was added to the reaction mixture at room temperature leading to the
formation of a brown colored precipitate. The precipitate was filtered, washed with hexane and dried to give 0.52 g (73%) of 233 as a brown solid. TLC $R_f = 0.6$ (CHCl$_3$/MeOH, 10:1); mp $>300$ °C. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta = 1.26$ (s, 9H, C(CH$_3$)$_3$), 7.17–7.21 (m, 1H, Ar), 7.24–7.28 (m, 1H, Ar), 7.43–7.45 (m, 1H, Ar), 7.86–7.88 (m, 1H, Ar), 11.12 (s, 1H, NH, exch), 11.85 (s, 1H, NH, exch), 12.03 (s, 1H, NH, exch). This material was used without further purification for the next step.

$N$-(4-Chloro-9H-pyrimido[4,5-b]indol-2-yl)-2,2-dimethylpropanamide (234).

To a 50 mL round-bottomed flask was added 233 (56 mg, 0.17 mmol) and phosphorus oxychloride (15 mL). The mixture was stirred and heated to reflux for 4 h. The phosphoryl trichloride was removed by evaporation under reduced pressure using a vacuum aspirator. The resulting residue was cooled in an ice and water mixture and neutralized with ammonium hydroxide solution to yield a precipitate that was filtered and dried over P$_2$O$_5$. The filtrate was extracted with chloroform and dried over sodium sulfate. The dry precipitate and filtrate were combined and dried under vacuum to provide 35 mg (59%) of 234 as a brown solid. TLC $R_f = 0.65$ (CHCl$_3$/MeOH, 10:1); mp 234.1–234.6 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.24$ (s, 9H, C(CH$_3$)$_3$), 7.34–7.38 (m, 1H, Ar), 7.53–7.55 (m, 2H, Ar), 8.15–8.17 (m, 1H, Ar), 10.25 (s, 1H, 2-NH, exch), 12.55 (s, 1H, 9-NH, exch). This material was used without further purification for the next step.
General procedure for the synthesis of \(N^4\)-substituted-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamines 1e and 2e:

To a 100 mL round-bottomed flask were added 234 (1 equivalent), appropriate aryl amine (3 equivalents), \(n\)-butanol and catalytic amount of conc. HCl. The resulting mixture was heated to reflux until the TLC showed completion of the reactant 234. The solvent was removed under reduced pressure to obtain a mixture that was dissolved in methanol, basified with ammonia in methanol, and silica gel was added. The solvent was removed by evaporation to yield a dry plug which was purified by flash chromatography using 1% methanol in chloroform. Fractions containing the desired product (TLC) were pooled and evaporated to obtain compounds 1e and 2e.

\(N^4\)-Ethyl-\(N^4\)-(4-methoxyphenyl)-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (1e).

Using the general procedure described above, compound 234 (80 mg, 0.26 mmol), \(N\)-ethyl-4-methoxyaniline 235 (120 mg, 0.78 mmol) and 1 drop of conc. HCl were heated to reflux in 50 mL of \(n\)-butanol for 72 h to afford 30 mg (31%) of 1e as a white solid. TLC \(R_f = 0.50\) (CHCl\(_3\)/MeOH, 15:1); mp 223.9–224.6 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 1.15–1.19\) (t, 3H, \(J = 6.9\) Hz, CH\(_3\)), 3.74 (s, 3H, OCH\(_3\)), 4.09–4.13 (q, 2H, \(J = 6.9\) Hz, \(N\)-CH\(_2\)), 5.66–5.68 (d, 1H, Ar), 6.15 (s, 2H, 2-NH\(_2\), exch), 6.50–6.54 (t, 1H, Ar), 6.90–6.97 (m, 3H, Ar), 7.12–7.16 (m, 3H, Ar), 11.20 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C\(_{19}\)H\(_{19}\)N\(_5\)O\_0.90CH\(_3\)OH: C, 65.98; H, 6.29; N, 19.33. Found: C, 65.81; H, 6.15; N, 19.31.
4-(6-Methoxy-3,4-dihydroquinolin-1(2H)-yl)-9H-pyrimido[4,5-b]indol-2-amine (2e).

Using the general procedure described above, compound 234 (80 mg, 0.26 mmol), 6-methoxy-1,2,3,4-tetrahydroquinoline 227 (120 mg, 0.78 mmol) and 1 drop of conc. HCl were heated to reflux in 30 mL of n-butanol for 60 h to provide 24.5 mg (26%) of 2e as an yellow solid. TLC $R_f = 0.48$ (CHCl$_3$/MeOH, 15:1); mp 172.8–174.0 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.95$–2.01 (m, 2H, $J = 6.2$ and 6.6 Hz, CH$_2$), 2.84–2.87 (t, 2H, $J = 6.6$ Hz, CH$_2$), 3.69 (s, 3H, OCH$_3$), 3.87–3.90 (t, 2H, $J = 6.2$ Hz, N-CH$_2$), 6.19–6.21 (d, 1H, Ar), 6.26 (s, 2H, 2-NH$_2$, exch), 6.46–6.48 (m, 1H, Ar), 6.57–6.58 (m, 1H, Ar), 6.71–6.74 (t, 1H, Ar), 6.868–6.874 (m, 1H, Ar), 7.04–7.07 (t, 1H, Ar), 7.21–7.23 (m, 1H, Ar), 11.31 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{20}$H$_{19}$N$_5$O·0.40CH$_3$OH: C, 68.39; H, 5.79; N, 19.55. Found: C, 68.20; H, 5.55; N, 19.53.

$N^4$-(4-Ethoxyphenyl)-$N^4$-methyl-9H-pyrimido[4,5-b]indole-2,4-diamine (3e).

In a 100 mL round-bottomed flask, compound 234 (80 mg, 0.26 mmol), 4-ethoxy-N-methylaniline 236 (120 mg, 0.78 mmol) and 1 drop of conc. HCl in 30 mL isopropanol were heated to reflux for 120 h. After cooling to rt, silica gel (500 mg) was added, and isopropanol was removed under reduced pressure. The dry residue was dissolved in methanol, basified with ammonia in methanol, and silica gel was added. The solvent was removed by evaporation to yield a silica gel plug. The plug was loaded on top of a silica gel column in chloroform (the weight of the silica was 15 times that of the plug). Purification was performed by flash chromatography using 0.5% methanol in chloroform and the fractions containing the product (TLC) were pooled and the solvent was evaporated to afford to provide 63 mg (72%) of 3e$^{178}$ as a white solid. TLC $R_f = 0.43$.}

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(CHCl₃/MeOH, 15:1); mp 238.8–239.6 °C. ¹H NMR (400 MHz, DMSO-d₆): δ = 1.29–1.32 (t, 3H, J = 6.9 Hz, CH₃), 3.50 (s, 3H, N-CH₃), 3.97–4.02 (q, 2H, J = 6.9 Hz, CH₂), 5.76–5.78 (d, 1H, Ar), 6.22 (s, 2H, 2-NH₂, exch), 6.53–6.57 (t, 1H, Ar), 6.88–6.90 (m, 2H, Ar), 6.95–6.98 (t, 1H, Ar), 7.13–7.15 (m, 3H, Ar), 11.24 (s, 1H, 9-NH, exch).

Elemental analysis calculated (%) for C₁₉H₉N₅O: C, 68.45; H, 5.74; N, 21.01. Found: C, 68.40; H, 5.61; N, 21.01.

N⁴-Methyl-N⁴-(4-(methylthio)phenyl)-9H-pyrimido[4,5-b]indole-2,4-diamine (1f).

In a round bottom flask, compound 234 (110 mg, 0.36 mmol), N-methyl-4-(methylthio)aniline 237 (223 mg, 1.45 mmol) and 2 drops of conc. HCl in 50 mL of n-butanol were heated to reflux for 48 h. After cooling to rt, silica gel (500 mg) was added, and isopropanol was removed under reduced pressure. The dry residue was dissolved in methanol, basified with ammonia in methanol, and silica gel was added. The solvent was removed by evaporation to yield a silica gel plug. Purification was performed by flash chromatography using 0.5% methanol in chloroform and the fractions containing the product (TLC) were pooled and the solvent was evaporated to afford to provide 86 mg (72%) of 1f as an off-white solid. TLC Rₚ = 0.44 (CHCl₃/MeOH, 15:1); mp 248.2–248.8 °C. ¹H NMR (400 MHz, DMSO-d₆): δ = 2.44 (s, 3H, SCH₃), 3.54 (s, 3H, NCH₃), 5.90–5.92 (d, 1H, Ar), 6.23 (s, 2H, 2-NH₂, exch), 6.57–6.59 (t, 1H, Ar), 6.99–7.03 (m, 1H, Ar), 7.12–7.14 (m, 2H, Ar), 7.17–7.19 (m, 1H, Ar), 7.22–7.24 (m, 2H, Ar), 11.31 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C₁₈H₁₇N₅S: C, 64.45; H, 5.11; N, 20.88; S, 9.56. Found: C, 64.22; H, 5.18; N, 20.88; S, 9.38.
**Ethyl 2-(2-nitrophenyl)-2-cyanoacetate (239).**

To an ice cold solution of ethyl cyanoacetate (14.36 g, 126.94 mmol) in anhydrous THF (170 mL) under argon atmosphere, was added potassium tert-butoxide (14.24 g, 126.94 mmol). Thus formed white suspension was stirred for 15 min and then 1-chloro-2-nitro-benzene 238 (10.00 g, 63.47 mmol) was added. The suspension was heated to reflux for 48 h after which 200 mL water was added and acidified to pH 2 with conc. HCl. The mixture was extracted with ether (250 mL × 3) and then the combined organic phase was dried (anhydrous sodium sulfate) and concentrated to give a dark yellow colored liquid. Flash chromatography using 10:1 hexane:ethyl acetate in a column packed with silica gel, four times the weight of the dark oil, provided 12.75 g (86%) of 239 as a viscous yellow liquid. TLC $R_f = 0.32$ (hexane/EtOAc, 3:1). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 1.23$–1.26 (t, 3H, CH$_3$), 4.16–4.24 (q, 2H, CH$_2$), 5.58 (s, 1H, CH), 7.58–7.62 (m, 1H, Ar), 7.68–7.72 (m, 2H, Ar), 8.15–8.17 (d, 1H, Ar). $^1$H NMR agreed well with the literature reported$^{129}$ values.

**Ethyl 2-amino-1H-indole-3-carboxylate (18).**

Compound 239 (12.75 g, 54.44 mmol) in 230 mL glacial acetic acid, was treated with a single charge of 8.5 g of zinc dust. The mixture was heated at 55 °C for 2 h and 4 g of Zn dust was added. After stirring at 55 °C for another 4 h, the reaction mixture was cooled and filtered through a pad of Celite. The pad was washed with acetic acid and the filtrate was concentrated to a residue that was neutralized with saturated Na$_2$CO$_3$ solution. The precipitate was filtered, dried over P$_2$O$_5$ and was dissolved in acetone. To this solution was added 15 g silica gel and the solvent was removed under reduced pressure to afford a
silica gel plug. Purification was done by flash chromatography eluting with 1% methanol in CHCl₃. Fractions containing the product (TLC) were pooled and evaporated to give 6.67 g (60%) of 18 as an off-white solid. TLC R_f = 0.65 (CHCl₃/MeOH, 10:1); mp 178.9–180.1 °C (lit.²⁰⁸ 179.0–181.0 °C). ¹H NMR (400 MHz, DMSO-d₆): δ = 1.31–1.34 (t, 3H, CH₃), 4.20–4.25 (q, 2H, CH₂), 6.67 (s, 2H, 2-NH₂, exch), 6.86–6.88 (t, 1H, Ar), 6.89–6.97 (t, 1H, Ar), 7.10–7.12 (d, 1H, Ar), 7.54–7.56 (d, 1H, Ar), 10.64 (s, 1H, 1-NH, exch). Elemental analysis calculated (%) for C₁₁H₁₂N₂O₂: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.64; H, 6.08; N, 13.66. ¹H NMR agreed well with the literature reported²⁰⁸ values.

2-[(1-Iminoethyl)amino]-1H-indole-3-carboxylic acid ethyl ester, hydrochloride (21).

Dry HCl gas was bubbled into a suspension of 18 (1.0 g, 4.9 mmol) and CH₃CN (65 mL) for 1.5 h. The mixture was then heated to reflux for 2.5 h and then cooled to 25 °C. The precipitate was collected and dried to give 1.2 g of 21 as a white hygroscopic solid. mp >300 °C (lit.¹³⁰ >300 °C). ¹H NMR (400 MHz, DMSO-d₆): δ = 1.32-1.36 (t, 3H, CH₃, J = 7.1 Hz), 2.43 (br, 3H, CH₃), 4.26–4.32 (q, 2H, CH₂, J = 7.1 Hz), 7.24–7.32 (m, 2H, Ar), 7.49–7.51 (d, 1H, Ar, J = 7.56 Hz), 8.04–8.06 (d, 1H, Ar, J = 7.51 Hz), 9.0 (br, 1H, 2-NH), 10.05 (br, 1H, 1-NH, exch), 12.06 (br, 1H, 1'-NH, exch), 12.75 (br, 1H, HCl, exch). ¹H NMR agreed well with the literature reported¹³⁰ values. This material was used directly for the next step without further purification.

2-Methyl-3H-pyrimido[4,5-b]indol-4(9H)-one (23).

A solution of 21 (1.2 g, 4.3 mmol), EtOH (350 mL), and 10% aq NaOH (35 mL) was
heated to reflux for 6 h. The cooled solution was concentrated to a residue that was
diluted with 25 mL of H₂O. The solution was adjusted to pH 2 with 3N HCl solution and
then stored at 25 °C for 30 min. The precipitated solid was collected, dissolved in
methanol and then was added 2.5 g of silica gel. The solvent was removed under reduced
pressure to provide a silica gel plug, which was purified by flash chromatography eluting
with 2% and 3% methanol in chloroform. Fractions containing the product (TLC) were
pooled and evaporated to give 0.96 g (77% over two steps) of 23 as a white solid. TLC Rf
= 0.51 (CHCl₃/MeOH, 10:1); mp >275 °C (lit.¹³⁰ >275 °C). ¹H NMR (400 MHz, DMSO-
⁶d₆): δ = 2.41 (s, 3H, 2-CH₃), 7.18–7.22 (t, 1H, Ar), 7.27–7.31 (t, 1H, Ar), 7.43–7.45 (d,
1H, Ar), 7.93–7.94 (d, 1H, Ar), 11.98 (s, 1H, 3-NH, exch), 12.12 (s, 1H, 9-NH, exch).
Elemental analysis calculated (%) for C₁₁H₉N₃O·0.39CH₃OH: C, 64.62; H, 5.03; N,
19.85. Found: C, 64.73; H, 4.88; N, 19.64. ¹H NMR agreed well with the literature
reported¹³⁰ values.

**4-Chloro-2-methyl-9H-pyrimido[4,5-b]indole (240).**

In a 250 mL round-bottomed flask was added 23 (1.04 g, 4.9 mmol) and 150 mL of
POCl₃. The reaction mixture was heated at 120 °C for 5.5 h. The POCl₃ was evaporated
and the mixture was neutralized using aqueous NH₄OH solution leading to a precipitate.
The precipitate was filtered, washed with cold water, dried and was dissolved in
methanol. To the solution was added silica and solvent was removed under reduced
pressure to provide a silica gel plug. The plug was purified by flash chromatography
using chloroform and 1% methanol in chloroform as the eluent. Fractions containing the
product (TLC) were pooled and evaporated to give 650 mg (79%) of 240 as an off-white
solid. TLC $R_f = 0.69$ (CHCl$_3$/MeOH, 10:1); mp 286.0–286.4 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.69$ (s, 3H, 2-CH$_3$), 7.38–7.42 (m, 1H, Ar), 7.57–7.62 (m, 2H, Ar), 8.22–8.23 (d, 1H, Ar), 12.56 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{11}$H$_8$ClN$_3$: C, 60.70; H, 3.70; N, 19.31; Cl, 16.29. Found: C, 60.68; H, 3.81; N, 19.11; Cl, 16.35.

$N$-(4-Methoxyphenyl)-$N$,2-dimethyl-$9H$-pyrimido[4,5-$b$]indol-$4$-amine (2f).

To a round-bottomed flask were added 240 (120 mg, 0.55 mmol), 4-methoxy-$N$-methylaniline 241 (302.5 mg, 2.2 mmol), 2 drops of conc. HCl and 25 mL of $n$-butanol. The reaction mixture was heated to reflux for 72 h. After cooling to rt, silica gel (500 mg) was added, and $n$-butanol was removed under reduced pressure to afford a silica gel plug. Flash chromatography was done using 0.5% and 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 137 mg (78%) of 2f as an off-white solid. TLC $R_f = 0.82$ (CHCl$_3$/MeOH, 5:1); mp 239.1–239.9 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.59$ (s, 3H, 2-CH$_3$), 3.59 (s, 3H, OCH$_3$), 3.75 (s, 3H, NCH$_3$), 5.89–5.90 (d, 1H, C5-CH), 6.66–6.69 (t, 1H, Ar), 6.92–6.94 (d, 2H, Ar), 7.14–7.17 (m, 1H, Ar), 7.19–7.21 (d, 2H, Ar), 7.31–7.33 (d, 1H, Ar), 11.85 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{19}$H$_{18}$N$_4$O: C, 71.68; H, 5.70; N, 17.60. Found: C, 71.91; H, 5.70; N, 17.69.

$3H$-Pyrimido[4,5-$b$]indol-$4(9H)$-one (242).

Compound 18 (400 mg, 1.96 mmol) and formamide (6 ml, 150 mmol) were taken in a 25 mL round bottom flask. The reaction mixture was stirred at 190 °C for 12 h. The reaction
mixture was cooled down, filtered, washed well with water and dried under reduced pressure. The dried precipitate was dissolved in 3 mL DMF and then precipitated using 30 mL Et₂O. The precipitate was collected, washed with Et₂O and dried over P₂O₅ to give 292 mg (73%) of 242 as a brown solid. TLC $R_f = 0.48$ (CHCl₃/MeOH, 5:1); mp >300 °C (lit.129 >300 °C). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.74$ (s, 0.3H, “DMF” N-CH₃), 2.89 (s, 0.3H, “DMF” NCH₃), 7.22–7.25 (m, 1H, Ar), 7.31–7.35 (m, 1H, Ar), 7.47–7.49 (d, 1H, Ar), 7.96 (s, 0.1H, “DMF” H), 7.98–8.00 (d, 1H, Ar), 8.12 (s, 1H, C2-CH), 12.19 (s, 1H, 3-NH, exch), 12.22 (br, 1H, 9-NH, exch). Elemental analysis calculated (%) for C₁₀H₇N₃O·0.2HCON(CH₃)₂: C, 63.72; H, 4.24; N, 22.43. Found: C, 63.70; H, 4.07; N, 22.16. $^1$H NMR agreed well with the literature reported129 values.

4-Chloro-9H-pyrimido[4,5-b]indole (243).

In a 100 mL round bottom flask was added 242 (340 mg, 1.70 mmol) and 35 mL of POCl₃. The reaction mixture was heated to reflux for 4 h. The POCl₃ was evaporated and the mixture was neutralized using aqueous NH₄OH solution leading to a precipitate. The precipitate was filtered, washed with cold water, dried and was dissolved in methanol. To the solution was added silica and solvent was removed under reduced pressure to provide a silica gel plug. Flash chromatography was done using chloroform and 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to give 310 mg (90%) of 243 as an off-white solid. TLC $R_f = 0.64$ (CHCl₃/MeOH, 10:1); mp >250 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 7.42–7.46$ (m, 1H, Ar), 7.64–7.65 (m, 2H, Ar), 8.29–8.31 (d, 1H, Ar), 8.79 (s, 1H, C2-CH), 12.80 (s, 1H, 9-NH, exch). $^1$H NMR agreed well with the literature reported129 values.
**N-(4-Methoxyphenyl)-N-methyl-9H-pyrimido[4,5-b]indol-4-amine (3f).**

To a 100 mL round bottom flask were added 243 (102 mg, 0.5 mmol), 4-methoxy-N-methylaniline 241 (274 mg, 2 mmol), 2 drops of conc. HCl and 30 mL of n-butanol. The reaction mixture was heated to reflux for 72 h. After cooling to rt, silica gel (500 mg) was added, and n-butanol was removed under reduced pressure. Purification was performed by flash chromatography using 1% methanol in chloroform as the eluent and the fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 58 mg (38%) of 3f as an off-white solid. TLC $R_f = 0.74$ (CHCl$_3$/MeOH, 10:1); mp >250 ºC. 

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 3.61$ (s, 3H, OCH$_3$), 3.76 (s, 3H, NCH$_3$), 5.95–5.96 (d, 1H, C5-CH), 6.70–6.73 (t, 1H, Ar), 6.93–6.95 (m, 2H, Ar), 7.19–7.23 (m, 3H, Ar), 7.36–7.37 (d, 1H, Ar), 8.57 (s, 1H, C2-CH), 12.07 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{18}$H$_{16}$N$_4$O: C, 71.04; H, 5.30; N, 18.41. Found: C, 70.92; H, 5.33; N, 18.24.

**General procedure for the synthesis of 5-(aryl sulfanyl)-9H-pyrimido[4,5-b]indole-2,4-diamines 1g–3g and 5g–7g.**

Compound 10 (50 mg, 0.21 mmol), the appropriate thiol (0.84 mmol), copper iodide (162 mg, 0.84 mmol), L-proline (98 mg, 0.84 mmol) and potassium carbonate (118 mg, 0.84 mmol) were added to a Biotage® microwave vial. Around 3.5–4 mL DMF was added as solvent and the tube was sealed. The reaction was run in a microwave at 180 ºC until the TLC showed completion of the reactant 10. After cooling to room temperature, the reaction mixture was transferred on top of a silica gel column and eluted with 1%, 2%,
3% and 4% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford \(1g\sim3g\) and \(5g\sim7g\) in 47–62% yield.

**2-Amino-5-(phenylsulfanyl)-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (1g).**

Using the general procedure described above, the reaction of \(\text{10}\) with benzene thiol \(\text{244}\) for 12 h afforded 39 mg (60%) of \(1g\) as a brown solid. TLC \(R_f = 0.39\) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp 247.0–247.4 °C (lit.\(^{193}\) 247 °C). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 6.51–6.54\) (d, 1H, C6-CH), 6.52 (br, 2H, 2-NH\(_2\), exch), 6.95–6.98 (t, 1H, \(J = 6.0\) Hz, C7-CH), 7.07–7.09 (d, 1H, \(J = 6.0\) Hz, C8-CH), 7.31–7.39 (m, 5H, Ar), 10.35 (s, 1H, 3-NH, exch), 11.55 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C\(_{16}\)H\(_{12}\)N\(_4\)OS\(\cdot0.23\)CH\(_3\)OH: C, 61.74; H, 4.12; N, 17.75; S, 10.16. Found: C, 61.50; H, 4.03; N, 17.81; S, 10.54. \(^1\)H NMR agreed well with the literature reported\(^{193}\) values.

**2-Amino-5-(2-naphthylsulfanyl)-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (2g).**

Using the general procedure described above, the reaction of \(\text{10}\) with naphthalene-2-thiol \(\text{245}\) for 4.5 h afforded 38 mg (50%) of \(2g\) as an off-white solid. TLC \(R_f = 0.47\) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp >250 °C (lit.\(^{193}\) >250 °C). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 6.54\) (br, 2H, 2-NH\(_2\), exch), 6.55–6.57 (d, 1H, C6-CH), 6.94–6.97 (t, 1H, C7-CH), 7.09–7.11 (d, 1H, C8-CH), 7.426–7.429 (d, 1H, Ar), 7.52–7.54 (m, 2H, Ar), 7.87–7.93 (m, 3H, Ar), 7.981–7.983 (d, 1H, Ar), 10.36 (s, 1H, 3-NH, exch), 11.58 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C\(_{20}\)H\(_{14}\)N\(_4\)OS\(\cdot0.6\)CH\(_3\)OH: C, 65.52; H, 4.38; N, 14.84; S, 8.49. Found: C, 65.31; H, 4.35; N, 14.99; S, 8.87. \(^1\)H NMR agreed well with the literature reported\(^{193}\) values.
2-Amino-5-(p-tolylthio)-3H-pyrimido[4,5-b]indol-4(9H)-one (3g).

Using the general procedure described above, the reaction of 10 with 4-methoxyphenyl thiol 246 for 16 h afforded 43 mg (62%) of 3g as a brown solid. TLC $R_f = 0.42$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp $>250$ °C (lit.$^{192}$ $>250$ °C). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 2.34$ (s, 3H, CH$_3$), 6.36–6.38 (d, 1H, C6-CH), 6.52 (br, 2H, 2-NH$_2$, exch), 6.90–6.93 (t, 1H, C7-CH), 7.01–7.03 (d, 1H, $J = 6.0$ Hz, C8-CH), 7.23–7.25 (d, 2H, Ar), 7.33–7.34 (d, 2H, Ar), 10.37 (s, 1H, 3-NH, exch), 11.54 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C$_{17}$H$_{14}$N$_4$O$_3$·0.3CH$_3$OH: C, 62.59; H, 4.61; N, 16.88; S, 9.66. Found: C, 62.24; H, 4.42; N, 17.00; S, 9.81. $^1$H NMR agreed well with the literature reported$^{209}$ values.

2-Amino-5-((4-methoxyphenyl)thio)-3H-pyrimido[4,5-b]indol-4(9H)-one (5g).

Using the general procedure described above, the reaction of 10 with 4-methoxyphenyl thiol 248 for 16 h afforded 39 mg (54%) of 97 as a brown solid. TLC $R_f = 0.47$ (CHCl$_3$/MeOH, 5:1 with 2 drops of conc. NH$_4$OH); mp $>250$ °C (lit.$^{192}$ $>250$ °C). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta = 3.81$ (s, 3H, CH$_3$), 6.23–6.24 (d, 1H, $J = 6.8$ Hz, C6-CH), 6.52 (br, 2H, 2-NH$_2$, exch), 6.87–6.91 (t, 1H, $J = 7.6$ Hz, C7-CH), 6.96–6.98 (d, 1H, $J = 6.8$ Hz, C8-CH), 7.02–7.05 (d, 2H, $J = 8.8$ Hz, Ar), 7.43–7.45 (d, 2H, $J = 8.8$ Hz, Ar), 10.40 (s, 1H, 3-NH, exch), 11.50 (s, 1H, 9-NH, exch). HPLC analysis: retention time = 27.99 min; peak area, 95.21%; eluent A, H$_2$O; eluent B, ACN; gradient elution (100% H$_2$O to 10% H$_2$O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt. $^1$H NMR agreed well with the literature reported$^{209}$ values.
2-Amino-5-((4-fluorophenyl)thio)-3H-pyrimido[4,5-b]indol-4(9H)-one (6g).

Using the general procedure described above, the reaction of 10 with 4-fluorophenyl thiol \(249\) for 16 h afforded 35 mg (51%) of \(95\) as a brown solid. TLC \(R_f = 0.47\) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp >300 °C (lit.\(^{192}\) >300 °C). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 6.42\) (d, 1H, \(J = 8.0\) Hz, C7-CH), 6.52 (br, 2H, 2-NH\(_2\), exch), 6.94 (t, 1H, \(J = 8.0\) Hz, C6-CH), 7.04–7.06 (m, 1H, C8-CH), 7.21–7.26 (m, 2H, Ar), 7.42–7.46 (m, 2H, Ar), 10.36 (s, 1H, 3-NH, exch), 11.54 (s, 1H, 9-NH, exch). \(^1\)H NMR agreed well with the literature reported\(^{209}\) values.

\[ \text{2-Amino-5-((4-phenoxyphenyl)thio)-3H-pyrimido[4,5-b]indol-4(9H)-one (7g).} \]

Using the general procedure described above, the reaction of 10 with 4-phenoxybenzene thiol \(250\) for 12 h afforded 40 mg (47%) of \(7g\) as a brown solid. TLC \(R_f = 0.46\) (CHCl\(_3\)/MeOH, 5:1 with 2 drops of conc. NH\(_4\)OH); mp >300 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 6.41–6.43\) (d, 1H, C6-CH); 6.56 (br, 2H, 2-NH\(_2\), exch); 6.94–6.98 (t, 1H, Ar); 7.02–7.05 (m, 3H, Ar); 7.09–7.11 (m, 2H, Ar); 7.18–7.21 (t, 1H, Ar); 7.42–7.46 (m, 4H, Ar); 10.40 (s, 1H, 3-NH, exch); 11.54 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for \(\text{C}_{22}\text{H}_{16}\text{N}_{4}\text{O}_{2}\text{S} \cdot 0.26\text{NH}_{4}\text{OH} \cdot 0.40\text{HCl}\): C, 62.30; H, 4.21; N, 14.07; S, 7.56; Cl, 3.35. Found: C, 62.30; H, 4.30; N, 14.04; S, 7.64; Cl, 3.73.

\[ \text{2-Amino-5-(phenylthio)-3H-pyrimido[4,5-b]indol-4(9H)-one hydrochloride (1g·HCl).} \]

Compound \(1g\) (50 mg) was dissolved in chloroform (2 mL), ethyl acetate (5 mL), methanol (0.2 mL) and diethyl ether (2 mL), and then HCl gas was bubbled into the solution till precipitation ceased. The yellow solid was collected by filtration and dried.
over P₂O₅ to afford 32 mg of 1g·HCl in 60% yield. Mp >300 °C. ¹H NMR (400 MHz, DMSO-ᴅ): δ = 6.52–6.54 (m, 1H, Ar), 6.60 (br, 3H, 2-NH₃⁺, exch), 6.98–7.00 (m, 1H, Ar), 7.11–7.13 (m, 1H, Ar), 7.38–7.39 (m, 5H, Ar), 10.61 (s, 1H, 3-NH, exch), 11.72 (s, 1H, 9-NH, exch). HRMS (ESI): m/z calculated for C₁₆H₁₃N₄O₅·HCl + H⁺ [M+H⁺]: 309.0810. Found: 309.0804. HPLC analysis: retention time = 24.80 min; peak area, 96.09%; eluent A, H₂O; eluent B, ACN; gradient elution (100% H₂O to 10% H₂O) over 60 min with a flow rate of 0.5 mL/min and detection at 254 nm; column temperature, rt.

2-Amino-5-(naphthalen-2-ylthio)-3H-pyrimido[4,5-b]indol-4(9H)-one hydrochloride (2g·HCl).

Compound 2g (50 mg) was dissolved in N,N-dimethylformamide (2 mL), dioxane (2 mL) and diethyl ether (20 mL), and then HCl gas was bubbled into the solution till precipitation ceased. The white solid was collected by filtration and dried over P₂O₅ to afford 30 mg of 2g·HCl in 59% yield. Mp >300 °C. ¹H NMR (400 MHz, DMSO-ᴅ): δ = 6.56–6.58 (m, 1 H, Ar), 6.70 (br, 3H, 2-NH₃⁺, exch), 6.95–6.99 (m, 1H, Ar), 7.12–7.14 (m, 1H, Ar), 7.41–7.43 (m, 1H, Ar), 7.52–7.55 (m, 2H, Ar), 7.87–7.99 (m, 4H, Ar), 10.59 (s, 1H, 3-NH, exch), 11.72 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C₂₀H₁₅ClN₄O₅·0.14C₄H₈O₂·0.05HCl: C, 60.37; H, 3.98; N, 13.70; Cl, 9.10; S, 7.84 Found: C, 60.19; H, 4.00; N, 13.88; Cl, 9.01; S, 7.78.
VI. SUMMARY

This dissertation describes the design and synthesis of following structural classes of compounds either as single agents with combination chemotherapy potential or as tubulin inhibitors or as selective *T. gondii* TS inhibitors:

1. 2,4-Diamino-5-thioaryl-9H-pyrimido[4,5-b]indoles
2. *N,N*-Disubstituted-5-methylfuro[2,3-\d]pyrimidin-4-amines
3. *N*\(^4\)-Aryl-5-chloro-2,4-diamino-pyrimido[4,5-b]indoles
4. 2,4-Disubstituted-9H-pyrimido[4,5-b]indole-4-amines
5. 2-Amino-4-oxo-5-thioaryl-9H-pyrimido[4,5-b]indoles

From these projects, a total of forty two novel compounds were synthesized and characterized, and among them, thirty six target compounds were submitted for various biological assays. In addition, five selective *T. gondii* TS inhibitors were resynthesized and submitted for further preclinical and X-ray crystallographic studies. These compounds are as follows:

1. 5-(4-Fluorophenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*1a*)
2. 5-(4-Trifluoromethylphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*2a*)
3. 5-(\(m\)-Tolylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*3a*)
4. 5-(o-Tolylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*4a*)
5. 5-(3,4-Dimethylphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*5a*)
6. 5-(3-Methoxyphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*6a*)
7. 5-(o-Methoxyphenylthio)-9H-pyrimido[4,5-b]indole-2,4-diamine (*7a*)
8. *N*-Ethyl-*N*-(4-methoxyphenyl)-5-methylfuro[2,3-\d]pyrimidin-4-amine (*1b*)
9. *N*-Isopropyl-*N*-(4-methoxyphenyl)-5-methylfuro[2,3-\d]pyrimidin-4-amine (*2b*)
10. \(N\)-([4-Methoxyphenyl]-5-methyl-N-propylfuro[2,3-\(d\)]pyrimidin-4-amine (3b)
11. \(N\)-Butyl-N-(4-methoxyphenyl)-5-methylfuro[2,3-\(d\)]pyrimidin-4-amine (4b)
12. \(N\)-Methyl-N-(4-ethylphenyl)-5-methylfuro[2,3-\(d\)]pyrimidin-4-amine (1c)
13. \(N\),5-Dimethyl-N-(4-(methylthio)phenyl)furo[2,3-\(d\)]pyrimidin-4-amine (2c)
14. \(N\),\(N\)-Dimethyl-\(N\)-([5-methylfuro[2,3-\(d\)]pyrimidin-4-yl]benzene-1,4-diamine (3c)
15. \(N\),\(N\),\(N\)-Trimethyl-\(N\)-([5-methylfuro[2,3-\(d\)]pyrimidin-4-yl]benzene-1,4-diamine (4c)
16. \(N\),5-Dimethyl-N-(4-nitrophenyl)furo[2,3-\(d\)]pyrimidin-4-amine (5c)
17. 5-Chloro-\(N\)-methyl-\(N\)-phenyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (1d)
18. 5-Chloro-\(N\)-methyl-\(N\)-\(p\)-tolyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (2d)
19. 5-Chloro-\(N\)-([4-chlorophenyl]-\(N\)-methyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (3d)
20. 5-Chloro-\(N\)-3-methoxyphenyl-\(N\)-methyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (4d)
21. 5-Chloro-\(N\)-3-fluorophenyl-\(N\)-methyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (5d)
22. 5-Chloro-4-([6-methoxy-3,4-dihydroquinolin-1(2\(H\)])-yl]-9\(H\)-pyrimido[4,5-\(b\)]indol-2-
    amine (6d)
23. \(N\)-Ethyl-\(N\)-([4-methoxyphenyl]-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (1e)
24. 4-[6-Methoxy-3,4-dihydroquinolin-1(2\(H\)])-yl]-9\(H\)-pyrimido[4,5-\(b\)]indol-2-amine (2e)
25. \(N\)-([4-Ethoxyphenyl]-\(N\)-methyl-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (3e)
26. \(N\)-Methyl-\(N\)-([4-(methylthio)phenyl]-9\(H\)-pyrimido[4,5-\(b\)]indole-2,4-diamine (1f)
27. \(N\)-([4-Methoxyphenyl]-\(N\),2-dimethyl-9\(H\)-pyrimido[4,5-\(b\)]indol-4-amine (2f)
28. \(N\)-([4-Methoxyphenyl]-\(N\)-methyl-9\(H\)-pyrimido[4,5-\(b\)]indol-4-amine (3f)
29. 2-Amino-5-([phenylsulfanyl]-3,9-dihydro-4\(H\)-pyrimido[4,5-\(b\)]indol-4-one (1g)
30. 2-Amino-5-([2-naphthylsulfanyl]-3,9-dihydro-4\(H\)-pyrimido[4,5-\(b\)]indol-4-one (2g)
During this study, a novel synthetic route was successfully optimized for the synthesis of target compounds 1a–7a. Also, it led to the resynthesis of gram quantities of lead compounds 1 and 2 with significant improvement in overall yield over the reported procedure. Ullmann coupling conditions were explored and successfully employed as a key step to obtain target compounds 2c–5c. Optimization of Ullmann coupling resulted in the resynthesis of 1g–3g, 5g and 6g with highly improved yields over previous methods.

Compounds 1a–7a, 1b–4b and 1c–5c were designed and synthesized as single agents which combine cytostatic mechanism (via RTK inhibition) with a cytotoxic mechanism (via TS or tubulin inhibition). Pyrimido[4,5-b]indoles 1a–7a were evaluated against multiple RTKs using whole cell assays and the 4'-CF₃ analog 2a and the 3',4'-
diMe analog 5a were the most potent inhibitors of VEGFR-2 and EGFR kinases. Furo[2,3-d]pyrimidines 1b–4b and 1c–5c were tested as inhibitors of tubulin and multiple RTKs (whole cell assays). Compounds 1b, 3b and 2c–4c had potent multi-RTK inhibition along with potent anti-tubulin activity, thus making them single agents with combination chemotherapy potential. Compounds 2b (N^4-iPr-4'-OMe) and 1c (N^4-Me-4'-Et) had potent RTK inhibition with weak microtubule depolymerizing activity. Therefore, compounds 2b and 1c can be used in anticancer chemotherapy either alone as RTK inhibitors or in combination chemotherapy with other cytotoxic agents.

4-Anilino-pyrimido[4,5-b]indoles 1d–6d, 1e–3e and 1f–3f were evaluated as tubulin inhibitors and as agents that can overcome Pgp and βIII-tubulin mediated drug resistance. Structural requirements of pyrimido[4,5-b]indoles for tubulin inhibition are: alkyl group at N^4-position, hydrogen at 5-position and alkoxy group at 4'-position.

2-Amino-4-oxo-5-thioaryl-pyrimido[4,5-b]indoles 1g–7g were tested in tgTS, hTS and T. gondii cell culture assays. Compounds 1g, 4g and 7g are nanomolar inhibitors of tgTS with up to a remarkable 122-fold selectivity for tgTS over hTS. These compounds are the only tgTS-selective inhibitors known till date and also provide a new target (TS) to combat T. gondii infection.

Pyrimido[4,5-b]indoles described in this dissertation represent one of the unique classes of molecules with diverse biological targets. 2-Amino-5-thioaryl-pyrimido[4,5-b]indoles with 4-amino substitution are anticancer agents which inhibit TS and multiple RTKs where as those with 4-oxo substitution were tgTS-selective antiparasitic agents. 4-Anilino-pyrimido[4,5-b]indoles are anticancer agents which inhibit tubulin and/or multiple RTKs.
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dihydrofolate reductase: Evidence for a TS intermediate and ts half-sites reactivity.


A. Biological evaluations

The biological evaluations of the compounds were performed by Dr. Susan Mooberry (tumor cell inhibitory assays, microtubule depolymerization assay and in vivo xenograft studies; Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229); Dr. Ernest Hamel (colchicine binding and bovine brain tubulin polymerization assays; 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. Michael Ihnat (RTK inhibitory assay, tumor cell inhibitory assays, CAM assay, in vivo xenograft and allograft studies; Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, Oklahoma City, OK 73117); Dr. Karen Anderson (tgTS inhibitory assay and X-ray crystallography studies; Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06511); Dr. Sherry F. Queener (DHFR inhibitory and T. gondii cell culture studies; Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202); and National Cancer Institute (60 cell tumor cell line screening assay and in vivo toxicity study; NCI, Developmental Therapeutics Program).

A.1 Biological data of 2,4-diamino-5-aryltio-9H-pyrimido[4,5-b]indoles

Table 7 shows inhibitory data for target compounds 1a–7a against EGFR, VEGFR-2 and PDGFR-β kinases. Kinase inhibition was performed in cell-based assays and therefore, it should be noted that cellular permeability might play a role in the activities
of these compounds. Whole cell assays were used for measuring RTK inhibition as these assays afford more meaningful results for translation to *in vivo* studies.

**Table 7.** Inhibition of RTKs by target compounds 1a–7a [Data kindly provided by Dr. Michael Ihnat, Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, OK 73117]

<table>
<thead>
<tr>
<th>Compd</th>
<th>Inhibition of</th>
<th>hTS (µM)</th>
<th>EGFR (nM)</th>
<th>VEGFR-2 (nM)</th>
<th>PDGFR-β (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (lead)</td>
<td>0.54</td>
<td>15.1 ± 2.5</td>
<td>22.6 ± 2.7</td>
<td>2.8 ± 0.3</td>
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</tr>
<tr>
<td>2 (lead)</td>
<td>0.39</td>
<td>10.4 ± 1.9</td>
<td>56.3 ± 8.2</td>
<td>40.3 ± 6.7</td>
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</tr>
<tr>
<td>1a</td>
<td>n.d.*</td>
<td>26.9 ± 5.2</td>
<td>38.6 ± 5.2</td>
<td>76.9 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>n.d.*</td>
<td>11.3 ± 1.7</td>
<td>22.0 ± 3.7</td>
<td>50.0 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>n.d.*</td>
<td>27.0 ± 4.2</td>
<td>38.2 ± 7.0</td>
<td>86.1 ± 10.7</td>
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</tr>
<tr>
<td>4a</td>
<td>n.d.*</td>
<td>23.6 ± 3.8</td>
<td>35.1 ± 6.6</td>
<td>73.1 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>n.d.*</td>
<td>12.6 ± 2.0</td>
<td>17.8 ± 1.9</td>
<td>55.8 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td>n.d.*</td>
<td>24.2 ± 3.8</td>
<td>42.5 ± 6.9</td>
<td>70.1 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* n.d. not determined.

The 4'-CF<sub>3</sub> analog 2a and 3',4'-dimethyl analog 5a were the most potent inhibitors against EGFR and VEGFR-2 kinases with activities comparable to the lead compound 1 where as 1a, 3a, 4a and 6a had 1.5-fold lower inhibition than 1 (Table 7). Compounds 3a and 4a with 3'- and 2'-methyl groups, respectively, displayed lower EGFR and VEGFR-2 kinase inhibition than the 4'-Me analog 2, thus indicating the importance of the methyl group at para-position for activity. The 4'-CF<sub>3</sub> analog 2a had a 2-fold increased VEGFR-
2 inhibition than the 4'-Me analog 2 and had activity comparable to the 4'-Cl analog 181. In this series of compounds, incorporation of strong electron-withdrawing groups at para-position of the thioaryl moiety gave higher VEGFR-2 potency while retaining EGFR inhibition. However, this improvement in activity cannot be entirely due to electron-withdrawing effect because the 4'-F analog 1a was less active than the 4'-H analog 1. The 3',4'-dimethyl analog 5a showed 3-fold higher anti-VEGFR-2 activity than the 4-Me analog 2. In the PDGFR-β inhibition assay, target compounds 1a–6a were 25–40-fold less active than the lead compound 1 (Table 7). The data indicates that substitution on the thioaryl ring can be detrimental to PDGFR-β kinase inhibition.

Lead compounds 1 and 2 were evaluated in MDA-MB-435 and 4T1 orthotopic breast cancer models.

**Figure 44.** Compounds 1 (NZ-43) and 2 (NZ-311) significantly decreased primary tumor growth and tumor vascular density in the MDA-MB-435 flank xenograft model. Human BLBCs, MDA-MB-435, were implanted into the lateral flank of NCr athymic nu/nu nude mice at 500,000 cells, and the mice were treated with carrier, pemetrexed, sunitinib, pemetrexed + sunitinib or 1 (25 mg/kg) or 2 (35 mg/kg) twice weekly until the end of the experiment. Tumor size was assessed by measuring tumor length, width, and depth twice
weekly using Vernier calipers. Tumor volume was calculated with the ellipsoid formula:
\[ \text{volume} = 0.52 \times \text{length} \times \text{width} \times \text{depth} \] and graphically represented as days after implantation. Statistics on these graphs were two way ANOVA with repeated measures post-test. [Data kindly provided by Dr. Michael Ihnat, Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, OK 73117]

In the MDA-MB-435 orthotopic xenograft model, compounds 1 (25 mg/kg) and 2 (35 mg/kg) significantly decreased tumor volume better than the combination of pemetrexed (30 mg/kg) and sunitinib (30 mg/kg) (Figure 44). Based on the remarkable potency compared to the combination of pemetrexed and sunitinib, compounds 1 and 2 are undergoing further preclinical evaluation.

Figure 45. Compounds 1 (NZ-43) and 2 (NZ-311) decreased primary tumor growth and lung metastases in the 4T1 orthotopic breast model. *, P < 0.05 by one way ANOVA with a Neuman–Keuls post-test. 4T1-Lucerase/GFP tagged cells were implanted orthotopically into BALB/c mice at 7500 cells, and the mice were treated with carrier, docetaxel, or 1 or 2 at their MTDs twice weekly until the end of the experiment. (A) Tumor volume was calculated with the ellipsoid formula: volume = 0.52 (length × width × depth). (B) After 32 days, animals were euthanized and lungs were excised and
immediately imaged at 25× using the LumaScope fluorescent imaging system. The number of metastases in both lungs was counted visually and represented graphically. [Data kindly provided by Dr. Michael Ihnat, Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, OK 73117]

Compounds 1 (25 mg/kg) and 2 (35 mg/kg) decreased tumor volume better than or equal to docetaxel (35 mg/kg) in the 4T1 triple negative orthotopic allograft model (Figure 45A). Also, compounds 1 and 2 significantly reduced tumor metastases compared to docetaxel (Figure 45B). Reduction of tumor metastases is important because many cancer patients die of metastatic disease.

A.2 Biological data of 4-substituted 5-methyl-furo[2,3-d]pyrimidines

Compounds 1b–4b and 1c–5c were first evaluated for their ability to depolymerize microtubules (Table 8). These compounds were also tested for their antiproliferative effects against MDA-MB-435 cell lines using the sulforhodamine B (SRB) assay. Compounds 1b–4b with different alkyl groups at the N⁴-position had decreased microtubule depolymerizing activity and IC₅₀ values compared to the lead N⁴-methyl analog 182. The activity decreased with increased homologation and the N⁴-iPr analog 2b was 60-fold less active (IC₅₀) than the N⁴-Et 1b.

The N⁴-Me analogs 1c–5c with variations at the 4'-position were less active as microtubule depolymerizers than the lead 182. The 4'-ethyl analog 1c had 30-fold decreased activity than the 4'-methoxy 182, indicating the importance of a hydrogen bond acceptor at 4'-position. The 4'-SMe analog 2c, 4'-NHMe analog 3c and 4'-NMe₂ analog 4c, respectively, had 2-, 10- and 6-fold decreased microtubule depolymerizing activity than the 4'-OMe analog 182. In addition, compounds 2c–4c had 3–18-fold lesser activity
in the MDA-MB-435 cell line. This data indicates that bioisosteric replacement of 4'-OMe group is detrimental to tubulin inhibitory and antiproliferative activities. The 4'-NO$_2$ analog 5c, as expected, had no activity in the microtubule depolymerization assay. $N^4$-Desmethyl intermediates 213–215, 218 and 219 were also tested for microtubule depolymerization and were inactive with EC$_{50}$ > 10 μM (Table 8).

**Table 8.** Antiproliferative and microtubule depolymerizing effects of target compounds 1b–4b and 1c–5c and intermediates 213–215, 218 and 219 [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC$_{50}$ ± SD (nM) (MDA-MB-435)</th>
<th>EC$_{50}$ (nM) for microtubule depolymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>182 (lead)</td>
<td>4.3 ± 0.3</td>
<td>23.9 ± 5.6</td>
</tr>
<tr>
<td>1b</td>
<td>8.1 ± 0.47</td>
<td>53</td>
</tr>
<tr>
<td>2b</td>
<td>504 ± 28</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>3b</td>
<td>27.3 ± 4.5</td>
<td>310</td>
</tr>
<tr>
<td>4b</td>
<td>100.3 ± 6.5</td>
<td>340</td>
</tr>
<tr>
<td>1c</td>
<td>183.1 ± 12.7</td>
<td>750</td>
</tr>
<tr>
<td>2c</td>
<td>12.3 ± 0.9</td>
<td>45</td>
</tr>
<tr>
<td>3c</td>
<td>73.7 ± 2.7</td>
<td>240</td>
</tr>
<tr>
<td>4c</td>
<td>43.8 ± 4.2</td>
<td>140</td>
</tr>
<tr>
<td>5c</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>213</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>214</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>215</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>218</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>219</td>
<td>n.d.</td>
<td>EC$_{50}$ &gt; 10 μM</td>
</tr>
<tr>
<td>CA4</td>
<td>4.4 ± 0.46</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*n.d. not determined.*
Table 9. Effects of 1b–4b and 2c on tubulin assembly and colchicine binding [Data kindly provided by Dr. Ernest Hamel, Division of Cancer Treatment and Diagnosis, Frederick National Laboratory for Cancer Research, National Cancer Institute, MD 21702]

<table>
<thead>
<tr>
<th>Compd</th>
<th>Inhibition of tubulin assembly (IC_{50} ± SD (µM))</th>
<th>Inhibition of colchicine binding (% inhibition ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 µM</td>
</tr>
<tr>
<td>182 (lead)</td>
<td>3.3 ± 0.5</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>1b</td>
<td>0.97 ± 0.09</td>
<td>84 ± 0.7</td>
</tr>
<tr>
<td>2b</td>
<td>3.0 ± 0.2</td>
<td>47 ± 0.2</td>
</tr>
<tr>
<td>3b</td>
<td>1.1 ± 0.1</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>4b</td>
<td>1.3 ± 0.2</td>
<td>61 ± 0.4</td>
</tr>
<tr>
<td>2c</td>
<td>1.2 ± 0.007</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>CA4</td>
<td>0.96 ± 0.07</td>
<td>90 ± 1</td>
</tr>
</tbody>
</table>

Based on their microtubule depolymerizing activities, compounds 1b–4b, and 2c were tested for their direct effects on tubulin assembly and colchicine binding (Table 9). Except 2b, all compounds, at 5 µM, inhibited [\(^3\)H]-colchicine binding to the protein comparable to CA4. Compounds 1b (N^4-Et), 3b (N^4-Pr) and 4b (N^4-Bu) inhibited tubulin assembly about as well as CA4 and about 3-fold better than 182 (N^4-Me).

Compounds 1b, 3b and 4b, at 5 µM concentration, also inhibited binding of [\(^3\)H]-colchicine by 87–95%, whereas the N^4-isopropyl analog 2b showed only 47% inhibition (Table 9). The lower activity might be due to steric hindrance in the colchicine binding site. The data indicates that the N^4-alkylation is tolerated for tubulin inhibition. Compound 2c (4'-SMe) inhibited tubulin assembly comparable to CA4 and is 3-fold better than the lead 4'-OMe analog 182.
Table 10. RTK inhibitory activities of target compounds 1b–3b and 1c–5c and intermediates 214, 215, 218 and 219 [Data kindly provided by Dr. Michael Ihnat, Department of Pharmaceutical Sciences, University of Oklahoma College of Pharmacy, OK 73117]

<table>
<thead>
<tr>
<th>Compd</th>
<th>EGFR (nM)</th>
<th>VEGFR-2 (nM)</th>
<th>PDGFR-β (nM)</th>
<th>A431 Cytotoxicity (nM)</th>
<th>CAM angiogenesis inhibition (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>182 (lead)</td>
<td>15.5 ± 2.0</td>
<td>9.3 ± 0.72</td>
<td>12.3 ± 2.0</td>
<td>5.1 ± 0.56</td>
<td>n.d.</td>
</tr>
<tr>
<td>1b</td>
<td>92.5 ± 9.9</td>
<td>67.3 ± 6.9</td>
<td>51.4 ± 7.2</td>
<td>3.4 ± 3.3</td>
<td>0.812 ± 0.091</td>
</tr>
<tr>
<td>2b</td>
<td>10.1 ± 0.91</td>
<td>23.4 ± 4.1</td>
<td>42.6 ± 5.6</td>
<td>4.8 ± 3.1</td>
<td>0.480 ± 0.078</td>
</tr>
<tr>
<td>3b</td>
<td>7.1 ± 0.74</td>
<td>12.0 ± 1.6</td>
<td>38.9 ± 6.6</td>
<td>2.3 ± 2.5</td>
<td>0.301 ± 0.044</td>
</tr>
<tr>
<td>1c</td>
<td>4.0 ± 0.39</td>
<td>7.8 ± 0.94</td>
<td>28.9 ± 4.0</td>
<td>1.4 ± 0.5</td>
<td>0.287 ± 0.040</td>
</tr>
<tr>
<td>2c</td>
<td>7.2 ± 0.81</td>
<td>13.6 ± 2.0</td>
<td>62.2 ± 9.6</td>
<td>2.9 ± 3.0</td>
<td>0.520 ± 0.051</td>
</tr>
<tr>
<td>3c</td>
<td>6.4 ± 0.97</td>
<td>12.7 ± 1.8</td>
<td>57.8 ± 9.9</td>
<td>2.9 ± 3.4</td>
<td>0.707 ± 0.082</td>
</tr>
<tr>
<td>4c</td>
<td>3.1 ± 0.06</td>
<td>15.6 ± 2.2</td>
<td>63.0 ± 10.7</td>
<td>1.0 ± 0.1</td>
<td>0.653 ± 0.072</td>
</tr>
<tr>
<td>5c</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td>432.7 ± 30.7</td>
<td>1.058 ± 0.124</td>
</tr>
<tr>
<td>214</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td>60 ± 10.1</td>
<td></td>
</tr>
<tr>
<td>215</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>218</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>219</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semaxanib</td>
<td>12.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10.6</td>
<td>18.2 ± 2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>172.1 ± 19.4</td>
<td>18.9 ± 2.7</td>
<td>83.1 ± 10.1</td>
<td>n.d.</td>
<td>1.3 ± 0.07</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.2 ± 0.2</td>
<td>124.7 ± 18.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>29.1 ± 1.9</td>
</tr>
</tbody>
</table>

*a n.d. not determined.

Compounds 1b–3b and 1c–5c were evaluated for inhibition against EGFR, VEGFR-2 and PDGFR-β kinases using high-throughput phosphotyrosine ELISA (Table 10). Compounds 3b and 1c–4c inhibited EGFR kinase less than 5-fold compared to the standard EGFR kinase inhibitor erlotinib. The N4-Me-4'-N,N-diMe analog 4c is the most
potent EGFR kinase inhibitor with an IC\textsubscript{50} of 3.1 nM. With the exception of compound \textbf{1b}, the EGFR kinase inhibition data correlated very well with the A431 cytotoxicity data. Compounds \textbf{1b–3b} and \textbf{1c–4c} had single-digit nanomolar IC\textsubscript{50} values in EGFR-overexpressing A431 cells.

Compounds \textbf{2c}, \textbf{3c} and \textbf{1c–4c} showed VEGFR-2 kinase inhibition better than or equal to the standard VEGFR-2 kinase inhibitor sunitinib (Table 10). In PDGFR-\(\beta\) kinase assay, compounds \textbf{1b–3b} and \textbf{1c–4c} had better activity than the standard sunitinib. Antiangiogenic effects of target compounds were evaluated using chorioallantoic membrane (CAM) assay. Compounds \textbf{1b–3b} and \textbf{1c–4c} had submicromolar inhibition of angiogenesis and are more potent than multi-RTK inhibitor sunitinib. Among the target compounds, compounds \textbf{3b} and \textbf{1c} are the most potent compounds in the CAM assay, with potencies about 4-fold better than sunitinib. Compound \textbf{5c} with electron-withdrawing NO\textsubscript{2} group at the 4'-position showed no activity against EGFR, VEGFR-2 and PDGFR-\(\beta\) kinases. However, in the CAM assay, compound \textbf{5c} had potency comparable to sunitinib. \(N^4\)-Desmethyl intermediates \textbf{213–215, 218} and \textbf{219} were also tested for RTK inhibition and were inactive (IC\textsubscript{50} >200 nM) against VEGFR-2, PDGFR-\(\beta\) and EGFR kinases.

At the \(N^4\)-position, longer chain alkyl groups were favored for the inhibition of tubulin and EGFR kinase but were not tolerated for VEGFR-2 and PDGFR-\(\beta\) inhibition, indicating steric intolerance at the \(N^4\)-position for anti-VEGFR-2 and anti-PDGFR-\(\beta\) activity. At the 4'-position of furo\([2,3-d]\)pyrimidines, both hydrogen-bond acceptor and hydrophobic group were required for inhibition of tubulin and EGFR kinase. However, for VEGFR-2 and PDGFR-\(\beta\) inhibition, hydrogen-bond acceptor at 4'-position was not
required. Compounds 1b, 3b and 2c–4c had potent multi-RTK inhibition along with potent anti-tubulin activity, thus making them single agents with combination chemotherapy potential. Compounds 2b (N^4-iPr-4'-OMe) and 1c (N^4-Me-4'-Et) showed potent RTK inhibition with weak microtubule depolymerizing activity. Therefore, compounds 2b and 1c can be used in anticancer chemotherapy either alone as RTK inhibitors or in combination chemotherapy with other cytotoxic agents.

**Table 11.** Target compounds 1b, 3b, 4b and 1c–4c circumvent Pgp and βIII-tubulin mediated resistance [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC_{50} ± SD (nM)</th>
<th>IC_{50} ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKOV3</td>
<td>SKOV3-MDR1-M6/6</td>
</tr>
<tr>
<td>182</td>
<td>7.7 ± 0.8</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>1b</td>
<td>14.5 ± 1.0</td>
<td>18.3 ± 1.0</td>
</tr>
<tr>
<td>3b</td>
<td>40.3 ± 2.6</td>
<td>71.0 ± 19</td>
</tr>
<tr>
<td>4b</td>
<td>147.8 ± 2.9</td>
<td>200.0 ± 17.1</td>
</tr>
<tr>
<td>1c</td>
<td>155.8 ± 15.0</td>
<td>161.9 ± 21.5</td>
</tr>
<tr>
<td>2c</td>
<td>18.6 ± 0.1</td>
<td>32.1 ± 1.5</td>
</tr>
<tr>
<td>3c</td>
<td>73.8 ± 1.8</td>
<td>85.6 ± 5.0</td>
</tr>
<tr>
<td>4c</td>
<td>70.3 ± 6.1</td>
<td>91.2 ± 19.5</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>5.0 ± 0.6</td>
<td>1,200 ± 58</td>
</tr>
<tr>
<td>CA4</td>
<td>5.5 ± 0.5</td>
<td>7.2 ± 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rr: Relative resistance

Potent microtubule depolymerizing agents 1b, 3b, 4b and 1c–4c were evaluated for Pgp-mediated drug resistance in an SKOV3 isogenic cell line pair (Table 11).<sup>210</sup> This cell line pair consists of the parental SKOV3 ovarian carcinoma cell and the Pgp-overexpressing SKOV3 MDR-1-6/6 cell line. The relative resistance value, designated
Rr, is obtained by dividing the IC\textsubscript{50} values obtained in the Pgp-expressing MDR-1-6/6 cells by the IC\textsubscript{50} obtained in the parental cells. A low Rr indicates that the cell lines have similar sensitivity to the compound and that the compound is able to overcome the expression of Pgp. It also suggests that the compound is a poor substrate for transport by Pgp. On the other hand, compound with a high Rr value indicates Pgp-mediated drug resistance. In this cell line pair, paclitaxel has a high Rr value of 240 (Table 11) while target compounds 1b, 3b, 4b and 1c–4c had low Rr values of less than 2, similar to that observed with CA-4. The data indicates that the target compounds circumvent Pgp-mediated drug resistance observed with taxanes and vinca alkaloids.

Compounds 1b, 3b, 4b and 1c–4c were also assessed for β\textsubscript{III}-tubulin mediated resistance in an isogenic HeLa cell line pair (Table 11). Rr value of a compound will be obtained by dividing the IC\textsubscript{50} value of the compound in the β\textsubscript{III}-tubulin expressing HeLa WTβ\textsubscript{III} cell line by the IC\textsubscript{50} value in the parental HeLa cell line. Paclitaxel showed β\textsubscript{III}-tubulin mediated resistance as it had an Rr value of 8.6. Compounds 1b, 3b, 4b and 1c–4c had an Rr value of \approx 1.0 implying equal sensitivity to the β\textsubscript{III}-expressing cell line. The data suggests that these compounds circumvent β\textsubscript{III}-tubulin mediated resistance associated with taxanes and vinca alkaloids.

The NCI performed the hollow fiber assay for lead compound 182. The hollow fiber assay provides an initial \textit{in vivo} experience for agents found to have reproducible activity in \textit{in vitro} anticancer assays. Tumor cell lines used in the hollow fiber assay are non-small cell lung carcinoma lines NCI-H23 and NCI-H522, breast carcinoma lines MDA-MB-231 and MDA-MB-435, colon sarcoma lines SW-620 and COLO 205, melanoma lines LOX and UACC-62, ovarian carcinoma lines OVCAR-3 and OVCAR-5,
and glioma lines U251 and SF-295. Compound 182 had an intraperitoneal (ip) score of 20 out of 48, subcutaneous (sc) score of 12 out of 48 and total score of 32 of 96. Based on the hollow fiber assay data, compound 182 was selected for xenograft studies in mice.

![Graph showing tumor volume over time](image)

**Figure 46.** Compound 182 (AG16) considerably decreased tumor volume in MDA-MB-435 tumor xenografts. [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

Compound 182 (AG16) was tested for antitumor effects in MDA-MB-435 tumor fragments (Figure 46). MDA-MB-435 tumor fragments were injected s.c. into the flank of nude mice. Once tumors reached ~200 mm³, mice were injected i.p. with 182 (60 mg/kg on days 1 and 3 and 50 mg/kg on day 9) or paclitaxel (20 mg/kg every other day until day 11). Three doses of 182 (60 mg/kg on days 1 and 3, and 50 mg/kg on day 9) led to highly significant inhibition of tumor growth superior to paclitaxel throughout the trial.

The NCI performed toxicity studies on 182 in the athymic nude mice. Compound 182 in 100% DMSO was administered i.p. on Day 1 and the mice were observed for survival for 15 days. Mice with the compound dose of 12.5, 25 and 50 mg/kg survived for 15 days. Higher compound doses of 100, 250 and 500 mg/kg resulted in the death of
mice after 4, 1 and 1 days respectively. Based on this data, compound 182 can be considered safe at doses ≤50 mg/kg in mice.

A.3 Biological data of N⁴-aryl-5-chloro-2,4-diamino-pyrimido[4,5-b]indoles

Compounds 1d–4d and 6d were evaluated for antiproliferative effects in MDA-MB-435 cell lines (Table 12). Compounds 1d–4d had >40-fold lower activity than the 4'-OMe analog 183. The data indicates that the methoxy group at 4'-position is important for antiproliferative activity. Compound 6d displayed 1.5-fold higher IC₅₀ than 183, indicating that conformational restriction of the N⁴-phenyl ring is not conducive to activity.

**Table 12.** Antiproliferative and microtubule depolymerizing effects of 1d–6d [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC₅₀ ± SD (µM) (MDA-MB-435)</th>
</tr>
</thead>
<tbody>
<tr>
<td>183 (lead)</td>
<td>0.183</td>
</tr>
<tr>
<td>1d</td>
<td>4.19 ± 0.5</td>
</tr>
<tr>
<td>2d</td>
<td>3.03 ± 0.3</td>
</tr>
<tr>
<td>3d</td>
<td>4.22 ± 0.5</td>
</tr>
<tr>
<td>4d</td>
<td>6.47 ± 0.3</td>
</tr>
<tr>
<td>5d</td>
<td>n.d. a</td>
</tr>
<tr>
<td>6d</td>
<td>0.271 ± 0.004</td>
</tr>
<tr>
<td>CA4</td>
<td>3.4 ± 0.6 nM</td>
</tr>
</tbody>
</table>

a n.d. not determined.

Lead compound 183 was evaluated by the NCI using hollow fiber assay,²¹¹ Compound 183 had an intraperitoneal (ip) score of 4 out of 48, subcutaneous (sc) score of
0 out of 48 and total score of 4 of 96. A compound is considered for xenograft testing if it has a combined ip + sc score of 20 or greater, a sc score of 8 or greater, or produces cell kill of any cell line at either dose level evaluated. Compound 183 (75 mg/kg) did not satisfy these criteria and hence was not selected for xenograft studies in mice.

**A.4 Biological data of 2,4-substituted pyrimido[4,5-b]indoles**

**Table 13.** Effects of 1e–3e and 1f–3f on proliferation of MDA-MB-435 cells and microtubule depolymerization in A-10 cells [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

<table>
<thead>
<tr>
<th>Compd</th>
<th><strong>IC$_{50}$ ± SD (nM)</strong> (MDA-MB-435)</th>
<th><strong>EC$_{50}$ (nM) for microtubule depolymerization</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>185 (lead)</td>
<td>14.7 ± 1.5</td>
<td>105 ± 12</td>
</tr>
<tr>
<td>1e</td>
<td>23.5 ± 1.2</td>
<td>198 ± 8</td>
</tr>
<tr>
<td>2e</td>
<td>54.4 ± 4.0</td>
<td>152 ± 2.8</td>
</tr>
<tr>
<td>3e</td>
<td>14.4 ± 0.5</td>
<td>83 ± 4</td>
</tr>
<tr>
<td>1f</td>
<td>89.1 ± 10.2</td>
<td>1100</td>
</tr>
<tr>
<td>2f</td>
<td>33.9 ± 3.4</td>
<td>136</td>
</tr>
<tr>
<td>3f</td>
<td>130.2 ± 7.8</td>
<td>1200</td>
</tr>
<tr>
<td>CA4</td>
<td>3.4 ± 0.6</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Among 2,4-substituted pyrimido[4,5-b]indoles 1e–3e and 1f–3f, compound 3e had the most antiproliferative and microtubule depolymerizing effects and was comparable or better than the lead compound 185 (Table 13). Conformationally restricted analog 2e had 3.5-fold higher IC$_{50}$ than 185, thus indicating that restriction of the N^4-phenyl ring is not
tolerated. Replacement of the 4'-OMe (185) with 4'-SMe group (1f) resulted in 6-fold increase in IC\textsubscript{50}. The 2-methyl 2f and the 2-H analog 3f displayed lower activity than the 2-amino 185 in both cell proliferation and microtubule depolymerizing assays indicating the importance of 2-amino group for antitubulin activity in this series of analogs.

**Table 14.** Compounds 1e–3e and 1f–3f: Inhibition of tubulin assembly and binding of [\textsuperscript{3}H]-colchicine [Data kindly provided by Dr. Ernest Hamel, Division of Cancer Treatment and Diagnosis, Frederick National Laboratory for Cancer Research, National Cancer Institute, MD 21702]

<table>
<thead>
<tr>
<th>Compd</th>
<th>Inhibition of tubulin assembly</th>
<th>Inhibition of colchicine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (\pm) SD ((\mu)M)</td>
<td>((%\text{ inhibition} \pm) SD) at (5 \mu\text{M})</td>
</tr>
<tr>
<td>185 (lead)</td>
<td>1.4 (\pm) 0.007</td>
<td>84 (\pm) 0.5</td>
</tr>
<tr>
<td>1e</td>
<td>1.7 (\pm) 0.2</td>
<td>76 (\pm) 3</td>
</tr>
<tr>
<td>2e</td>
<td>2.2 (\pm) 0.1</td>
<td>71 (\pm) 5</td>
</tr>
<tr>
<td>3e</td>
<td>1.7 (\pm) 0.07</td>
<td>82 (\pm) 1</td>
</tr>
<tr>
<td>1f</td>
<td>2.3 (\pm) 0.3</td>
<td>67 (\pm) 5</td>
</tr>
<tr>
<td>2f</td>
<td>1.2 (\pm) 0.04</td>
<td>67 (\pm) 5</td>
</tr>
<tr>
<td>3f</td>
<td>2.3 (\pm) 0.4</td>
<td>62 (\pm) 4</td>
</tr>
<tr>
<td>CA4</td>
<td>1.0 (\pm) 0.09</td>
<td>99 (\pm) 0.2</td>
</tr>
</tbody>
</table>

Target compounds 1e, 3e and 2f inhibited tubulin assembly comparable to CA4 (Table 14). In this series of compounds, compound 3e\textsuperscript{178} was the most potent in the colchicine displacement assay, with activity comparable to the lead compound 185.

Compounds 1e–3e and 1f–3f were evaluated for Pgp and \(\beta\)III-tubulin mediated resistance in isogenic SKOV3 and HeLa cell line pairs, respectively (Table 15).\textsuperscript{210} In SKOV3 cell line pair, paclitaxel has a high R\textsubscript{r} value of 590 indicating that the cells overexpressing Pgp were resistant to paclitaxel. Target compounds 1e–3e and 1f–3f had
Rr values of less than 2, similar to that observed with CA-4. Compound 3e\textsuperscript{178} had almost 2-fold lesser activity than CA4 in both wild type and Pgp-overexpressing SKOV3 cell lines.

**Table 15.** Compounds 1e–3e and 1f–3f are effective in cell lines expressing Pgp or βIII-tubulin [Data kindly provided by Dr. Susan Mooberry, Department of Pharmacology, University of Texas Health Science Center at San Antonio, TX 78229]

<table>
<thead>
<tr>
<th>Compd</th>
<th>IC\textsubscript{50} ± SD (nM)</th>
<th>IC\textsubscript{50} ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKOV3 MDR-1-6/6</td>
<td>HeLa</td>
</tr>
<tr>
<td>185 (lead)</td>
<td>27.6 ± 1.8</td>
<td>34.4 ± 5.9</td>
</tr>
<tr>
<td>1e</td>
<td>53.0 ± 1.9</td>
<td>72.0 ± 7.9</td>
</tr>
<tr>
<td>2e</td>
<td>83.2 ± 5.7</td>
<td>134.7 ± 23.2</td>
</tr>
<tr>
<td>3e</td>
<td>23.0 ± 1.1</td>
<td>29.1 ± 1.7</td>
</tr>
<tr>
<td>1f</td>
<td>156.4 ± 16.0</td>
<td>160.1 ± 14.9</td>
</tr>
<tr>
<td>2f</td>
<td>60.5 ± 2.4</td>
<td>78.0 ± 8.4</td>
</tr>
<tr>
<td>3f</td>
<td>173.2 ± 8.6</td>
<td>223.7 ± 21.2</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>4.4 ± 0.6</td>
<td>2596 ± 119</td>
</tr>
<tr>
<td>CA4</td>
<td>9.7± 0.2</td>
<td>11.5 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rr: Relative resistance

In the HeLa cell line pair, paclitaxel showed βIII-tubulin mediated resistance as it had an Rr value of 5.8 (Table 15) where as target compounds 1e–3e and 1f–3f had an Rr value of ≈1.0 implying equal sensitivity to the βIII-expressing cell line. Compound 3e\textsuperscript{178} displayed potency comparable to the lead compound 185 in HeLa and βIII-tubulin expressing cell lines. The data indicates that these compounds overcome Pgp and βIII-tubulin mediated resistance observed clinically with taxanes and Vinca alkaloids.

Lead compound 185 was evaluated using hollow fiber assay by the NCI\textsuperscript{211}. Compound 185 had an intraperitoneal (ip) score of 14 out of 48, subcutaneous (sc) score
of 2 out of 48 and total score of 4 of 96. Compound 185 was not considered for xenograft studies as it had a combined ip + sc score of less than 20, a sc score of less than 8, and did not produce cell kill of any cell line at dose level (150 mg/kg) evaluated.

A.5 Biological data of 2-amino-4-oxo-5-thioaryl-9H-pyrimido[4,5-b]indoles

Table 16. Kinetic evaluation of 1g, 2g, 4g and 7g reveals species selectivity for tgTS over hTS

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>Selectivity (hTS K&lt;sub&gt;i&lt;/sub&gt; / tgTS K&lt;sub&gt;i&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tgTS</td>
<td>hTS</td>
<td>tgTS</td>
</tr>
<tr>
<td>1g</td>
<td>23 ± 6.6</td>
<td>481 ± 162</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>2g</td>
<td>21 ± 13</td>
<td>28.8 ± 21</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>4g</td>
<td>35.8 ± 2.9</td>
<td>3250 ± 184</td>
<td>3.1 ± 0.25</td>
</tr>
<tr>
<td>7g</td>
<td>43.4 ± 5.2</td>
<td>738.7 ± 235.4</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>T. gondii TS–DHFR (25 nM) and human TS (50 nM) were preincubated with 100 µM dUMP and inhibitor. The reaction was initiated with 100 µM methyleneTHF.

Compounds 1g, 2g, 4g and 7g exhibited single-digit nanomolar K<sub>i</sub> values against tgTS (Table 16). More importantly, compound 1g, 4g and 7g, respectively, showed unprecedented 28-fold, 122-fold and 22-fold selectivity for tgTS over hTS.

To determine the structural basis for the observed activity and selectivity, X-ray co-crystal structures of 1g and 4g with T. gondii TS–DHFR was determined (Figure 47). The atomic coordinates of T.gondii TS–DHFR/compound 1g and 4g described here have been deposited in the PDB (www.pdb.org) with accession codes 4KY4 and 4KYA, respectively. Upon superimposing the tgTS and hTS, almost all of the residues in the active site were found to be identical. It was surprising that a remarkable 28-fold selectivity (K<sub>i</sub> value) was observed for 1g despite almost identical architectures of the active sites of tgTS and hTS.
Figure 47. Inhibitors 1g and 4g in the active site of *T. gondii* TS. The key residues are highlighted in red. The inhibitors 1g and 4g and the substrate dUMP are colored yellow.\(^{193}\)

As shown in Figure 47, the crystal structure of the tgTS–DHFR with 1g and 4g revealed extensive base stacking interactions between the inhibitor and the nucleotide dUMP as expected. The phenyl ring of 1g or the 1'-naphthyl ring of 4g are oriented almost at a right angle to the tricyclic scaffold and interacts with Ile402, Leu516, Phe520 and Met608. The scaffold is also stabilized by hydrophobic interactions of the C-ring with Trp403. Using X-ray co-crystal structure of 1g with tgTS–DHFR, ligand interactions were generated using MOE 2013.08\(^{184}\) (Figure 48).
Figure 48. Interactions between 1g and residues in the active site of tgTS.

Compound 1g is bound to the active site by aromatic stacking of the pyrimido[4,5-b]indole scaffold and the dUMP pyrimidine ring. Additionally, hydrogen bonds are formed between the N9 of 1g and carboxyl of Asn406, the N3 and the 2-NH$_2$ group with the –COOH of Asp513 (Figure 48). The 2-NH$_2$ group forms additional hydrogen bond with the backbone oxygen of Ala609.

Table 17. Toxoplasma cell culture study of 1g, 2g and 4g

<table>
<thead>
<tr>
<th>Condition</th>
<th>T. gondii in media at Day 4</th>
<th>T. gondii in cells at Day 4</th>
<th>Estimated IC$_{50}$ value, µM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>3710000</td>
<td>1140000</td>
<td></td>
</tr>
<tr>
<td>1g (5 µM)</td>
<td>100000</td>
<td>140000</td>
<td>3.7</td>
</tr>
<tr>
<td>2g (5 µM)</td>
<td>2350000</td>
<td>340000</td>
<td>2.6</td>
</tr>
<tr>
<td>4g (5 µM)</td>
<td>&lt;25000</td>
<td>&lt;2500</td>
<td>0.6</td>
</tr>
<tr>
<td>pyrimethamine (3 µM)</td>
<td>&lt;2500</td>
<td>&lt;2500</td>
<td>0.57</td>
</tr>
</tbody>
</table>

$^a$Dose response curves using data from the three concentrations of test compounds gave estimates of IC$_{50}$ values.
Compounds 1g, 2g and 4g have also been evaluated against T. gondii cells in culture (Table 17). The culture model requires that the experimental compounds rapidly kill exposed tachyzoites as they are released from cells and/or that the experimental compounds penetrate the cell and the vacuole containing tachyzoites where the compounds may kill the organism or prevent its replication. Compound 4g, at 5 µM, was found to be equivalent to pyrimethamine at 3 µM indicating a significant effect on T. gondii in culture comparable to a clinically used agent PM. Both compounds 1g and 2g, at 5 µM, were 4–6 times less active than pyrimethamine at 3 µM.

Methods for biological evaluation

**Effects of compounds on cellular microtubules.** A-10 cells were used to evaluate the effects of the compounds on cellular microtubules using indirect immunofluorescence techniques. Cells were treated for 18 h with compounds and microtubules were visualized with an antibody towards β-tubulin (Sigma–Aldrich Co.). EC$_{50}$ values were calculated from an average of a minimum of three independent experiments.

**Sulforhodamine B (SRB) assay.** The SRB assay was used to evaluate the antiproliferative and cytotoxic effects of the compounds against cancer cells. MDA-MB-435, SK-OV-3 and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). The IC$_{50}$ values (concentration required to cause 50% inhibition of proliferation) represent an average of 3 independent experiments using triplicate points in each experiment.
In vitro tubulin polymerization. The effect of the compounds on tubulin polymerization was measured using purified porcine brain tubulin (Cytoskeleton Inc.).\textsuperscript{55} Briefly, 2.2 mg/mL of purified porcine brain tubulin was incubated with tubulin polymerization buffer (80 mM Na-PIPES, pH 6.9, 1 mM EGTA, 1 mM MgCl\textsubscript{2}, 10 mM GTP and 10% glycerol) and 10 μM of each corresponding drug. The polymerization of tubulin was monitored turbidimetrically by measuring the absorbance at 340 nm at 37 °C in a SpectraMax 96-well plate spectrophotometer.

Quantitative tubulin studies. Bovine brain tubulin was purified as described previously.\textsuperscript{213} Briefly, 1.0 mg/mL of tubulin (10 μM) was incubated for 15 min with 0.8M monosodium glutamate (pH of 2M stock solution adjusted to 6.6 with HCl), varying compound concentrations and 4% (v/v) dimethyl sulfoxide (DMSO) as solvent. After preincubation, 0.4 mM GTP was added. The reaction mixtures were transferred to the cuvettes at 0 °C and were placed in a recording spectrophotometer equipped with an electronic temperature controller. After baselines were established, the temperature was jumped over about 30 s to 30 °C, and changes in turbidity were monitored for 20 min. The compound concentration that caused a 50% reduction in increase in turbidity, interpolated from the values obtained with defined compound concentrations, was defined as the IC\textsubscript{50} value. The assay to measure inhibition of [\textsuperscript{3}H]colchicine binding was described in detail previously.\textsuperscript{214} Briefly, 0.1 mg/mL (1.0 μM) tubulin was incubated, at 37 °C, 5.0 μM [\textsuperscript{3}H]colchicine, and potential inhibitors at 1.0 or 5.0 μM, as indicated. Incubation was for 10 min at which point the reaction has reached 40-60% of the maximum colchicine that can be bound in reaction mixtures without inhibitor. The
[^3H]colchicine was a product of Perkin–Elmer. CA4 was a generous gift of Dr. G. R. Pettit, Arizona State University.

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

**Phosphotyrosine enzyme–linked immunosorbent assay (ELISA).** A high-throughput phosphotyrosine ELISA was developed for evaluating the effect of compounds on RTKs. Cells used for these experiments have been shown to overexpress particular RTKs; specifically A431 for EGFR, U251 for VEGFR-2, and SH-SY5Y for PDGFR-β. Briefly, cells at 60–75% confluence are placed in serum-free medium for 18 h to reduce the background of 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 ½ Log increments by 100 ng/mL of purified growth factor (EGF, VEGF, or PDGF-BB) for 10 min. The reaction was stopped, and the cells were 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 (Sigma–Aldrich Co.). The TBS solution was then removed, and cells fixed to the plate for 30 min at 60 °C and further incubation in 70% ethanol for an additional 30 min. Cells were further exposed to block (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, 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 plate reader. The
known RTK-specific inhibitors (semaxanib, sunitinib, erlotinib) were used as positive controls for kinase inhibition. Data were graphed as a percent of cells receiving growth factor alone, and IC\textsubscript{50} values were calculated from two to three separate experiments (\(n = 8–24\)) using sigmoidal dose-response relations in Prism 3.0 software (GraphPad).

**Chorioallantoic membrane (CAM) assay.** The CAM assay is a standard assay for testing antiangiogenic agents and was performed as previously\textsuperscript{216} described. Briefly, fertile leghorn chicken eggs (CBT Farms, Chestertown, MD) were allowed to grow until 10 days of incubation. The pro-angiogenic factors, human VEGF-165 and basic fibroblast growth factor (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. To the same microbial disk, antiangiogenic compounds were then added 8 h after the VEGF/bFGF at saturation and embryos were allowed to incubate for an additional 40 h. After 48 h, 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 7.5× and 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 were counted as a measure of vascularity. Sunitinib and semaxanib were used as positive controls for antiangiogenic activity. Data were graphed as a percent of CAMs receiving bFGF/VEGF only and IC\textsubscript{50} values calculated from two to three separate experiments (\(n = 5–11\)) using non-linear regression dose-response relation analysis.
MDA-MB-435 flank tumor model. Human MDA-MB-435 basal-like breast cancer cells (500,000) in media were implanted into the lateral flank of 8 week old female NCr athymic nu/nu nude mice (Charles River, Wilmington, DE). Tumor sizes (length, width, depth) were measured twice weekly. When volumes reached 75–100 mm$^3$ (day 7 after implantation), the tumors were treated with drugs at their maximum tolerated dose (MTD) and tumor volumes were measured twice weekly. The MTD of docetaxel was found to be 35 mg/kg, sunitinib 30 mg/kg, pemetrexed 30 mg/kg, 1 25 mg/kg and 2 35 mg/kg.

4T1 triple negative breast orthotopic allograft model. 4T1-Luc2GFP dual luciferase/GFP tagged cells were purchased from Caliper Life Sciences (Hopkinton, MA) and maintained in Dulbecco’s modification of minimal essential media (DMEM) containing 10% Cosmic Calf Serum (Hyclone, Logan, UT). 750 cells [verified by fluorescence imaging to be >98% GFP positive and counted three times on a TC10 automated cell counter (BioRad, Hercules, CA)] in 100 µL PBS with 1 mM EDTA were implanted subcutaneously into the left fat pad #4 of 8 week old female BALBc/J mice using a tuberculin syringe. The MTD of drugs were delivered to animals twice weekly starting three days after implantation. Tumor sizes (length, width, depth) were measured three times weekly. At day 33 post implantation, animals were humanely euthanized using the AALAC approved method of carbon dioxide asphyxiation. Tumors and lungs were removed and fresh lungs imaged using a LumaScope fluorescent imaging system (Bulldog Bio, Portsmouth, NH) at 25× magnification with the number of metastases per lung counted by hand from captured images.
Expression, Purification, and crystallization of *T. gondii* TS-DHFR. Wild-type *T. gondii* TS–DHFR was expressed and purified as described previously.\textsuperscript{217} The loop truncated form of TS–DHFR enzyme was purified as described before for structure determination. Wildtype TS–DHFR was used for kinetic assays. Briefly, the TS–DHFR was overexpressed in *E. coli* BL21 cells after induction with IPTG. In the final step, the protein was stored in buffer containing 25 mM Tris pH 7.3 and 10 mM DTT. The ligands NADPH, methotrexate, dUMP and the inhibitor were added to a final concentration of 500 µM. Crystallization was achieved at a concentration of 10 mg/mL with a 1:1 ratio of the enzyme and the well solution containing PEG 3350 and potassium phosphate. The crystals were cryoprotected in mother liquor containing ethylene glycol and frozen in stepwise transfers into liquid nitrogen. Data were collected at beamlines X25 and X29 at Brookhaven National Laboratory.

**X-Ray structure determination.** Initial processing of X-ray data was accomplished by using HKL2000.\textsuperscript{218} The loop truncated model (PDB accession code: 4EIL) was used as the search model for molecular replacement by using PHASER.\textsuperscript{219} Refinement of the structure was carried out by REFMAC.\textsuperscript{220} The ligand and its topology file was generated by the PRODRG server,\textsuperscript{221} and manual adjustments to the model and the ligand were made in COOT.\textsuperscript{222} Figure was generated using PYMOL.\textsuperscript{223}

**IC\textsubscript{50} and K\textsubscript{i} values of *T. gondii* TS–DHFR.** Separately, the *T. gondii* TS–DHFR (25 nM) and human TS (50 nM) were incubated with dUMP and inhibitor both at 100 µM, and the reaction was initiated with methylene tetrahydrofolate (100 µM).\textsuperscript{193} The change in absorbance was monitored by TECAN plate reader at 340 nm. The data was plotted using KALEIDAGRAPH. The IC\textsubscript{50} values were then determined, from which K\textsubscript{i} was
determined. The nucleotide dUMP, the cofactor NADPH and methylene tetrahydrofolate were purchased from Sigma–Aldrich Co.

**T. gondii cell culture study.** *T. gondii* strain PHΔHX was grown in culture on an immortalized human cell line (human telomerase reverse transcriptase (H-tert)) both to prepare inoculum and for the experimental drug tests.\(^{193}\) Freshly lysed parasites were removed from culture, resuspended in sterile phosphate buffered saline, counted, and diluted in medium to allow addition of 1300 *T. gondii* tachyzoites to each well of 24-well culture plates containing monolayers of H-tert cells. After incubation for 4 h at 37 °C, each well received either 500 µL of media (DMEM, 1% fetal calf serum), or media including various concentrations (5, 0.5, 0.05 µM) of experimental drug. Wells were evaluated visually and one set of wells was harvested daily for four days. Media (10 µL) from harvested wells was spread over a 1 cm square area on a microscope slide; cells in harvested wells were scraped and resuspended in 100 µL of fresh medium and 10 µL was spread over a 1 cm square area on a microscope slide. After air-drying, each slide was fixed in methanol and stained with Giemsa Plus. Organisms and cells/1000× field were counted as an indication of growth of *T. gondii* in the presence or absence of test compounds or pyrimethamine, which served as the positive control. By day 2 of growth, *T. gondii* tachyzoites began to be detected in the sampled control cell monolayers. By day 4, untreated monolayers were mostly lysed with numerous tachyzoites in both media samples and cell samples.

**B. Bulk synthesis of lead compounds 182, 183, 185 and target compound 4g**

\(^{N^4}-\)Methylation of 181 using dimethyl sulfate furnished 182 in 70% yield (Scheme 48).\(^{174}\) This scheme was used to synthesize 2 g of 182 required for preclinical evaluation.
Scheme 48. Synthesis of 182

Scheme 49. Synthesis of 183

Nucleophilic displacement of the 4-chloro of 221 with N-methyl-4-methoxyaniline 241 followed by deprotection of the 2-amino group provided 183 in 60% yield (Scheme 49). This procedure was utilized to synthesize 250 mg of 183 required for hollow fiber assay.

Scheme 50. Synthesis of 185
Compound 234 was reacted with *N*-methyl-4-methoxyaniline 241 in *n*-butanol at reflux followed by base-mediated deprotection of the 2-amino group to afford 185 in 62% yield (Scheme 50). This procedure was employed to synthesize 500 mg of 185 in improved yield (62%) over the reported procedure (42%) in which the displacement was carried out in isopropanol at reflux for 72 h.

**Scheme 51. Synthesis of 4g**

Displacement of the 2-bromo of 192 with ethyl cyanoacetate furnished 251 (Scheme 51). Zinc-catalyzed reduction of the 2-nitro of 251 followed by cyclization provided the substituted indole 252. Cyclocondensation of 252 with carbamimidic chloride hydrochloride afforded 2-amino-4-oxo-5-bromo-pyrimido[4,5-*b*]indole 253. Reaction temperature should be maintained between 120–125 °C to prevent the formation of side products. Ullmann coupling of 253 and 1-naphthylthiol 247 furnished target compound 4g in 24% yield. Column chromatography of 4g should not be carried out using a CombiFlash® Rf system because 4g sticks to the silica gel and does not elute out.
Experimental section for Schemes 48–51.

\textit{N-(4-Methoxyphenyl)-N,5-dimethylfuro[2,3-d]pyrimidin-4-amine (182).}

To a 25 mL round bottom flask was weighed 181 (510 mg, 2 mmol) and was added DMF (10 mL) to afford a solution. The flask was purged with argon for 5 min followed by cooling down to 0 °C using ice bath. Sodium hydride (144 mg, 6 mmol) was added to the solution at 0 °C. The solution was stirred for 30 min at 0 °C under argon atmosphere. Dimethyl sulfate (757 mg, 6 mmol) was injected to the reaction mixture and the flask was warmed to room temperature. The mixture was stirred at room temperature for another 3 h at the end of which 1N HCl (5 mL) was added carefully to quench the reaction followed by water (10 mL) to afford a precipitate. The product was extracted with ethyl acetate (10 mL × 3). The combined organic extracts were washed with brine (10 mL), dried (anhydrous sodium sulfate) and concentrated under reduced pressure. Silica gel (2 g) was added and the solvent evaporated to obtain a plug. Column chromatography by elution with hexanes and ethyl acetate (5:1) afforded 182 (416 mg, 70%) as an off-white solid. TLC \(R_f = 0.87\) (CHCl₃/MeOH, 10:1); mp 87.0–87.8 °C (lit.¹⁷⁴ 84.8–85.6 °C); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 1.05\) (s, 3H, CH₃), 3.44 (s, 3H, OCH₃), 3.77 (s, 3H, NCH₃), 6.96–6.98 (d, 2H, Ar, \(J = 9.0\) Hz), 7.19–7.22 (d, 2H, Ar, \(J = 9.0\) Hz), 7.52 (s, 1H, C₆-CH), 8.45 (s, 1H, C₂-CH). Elemental analysis calculated (%) for C₁₅H₁₅N₃O₂: C, 66.90; H, 5.61; N, 15.60. Found: C, 66.92; H, 5.66; N, 15.65.
5-Chloro-\(N^4\)-(4-methoxyphenyl)-\(N^4\)-methyl-9\(H\)-pyrimido[4,5-b]indole-2,4-diamine (183).

Compound 221 (60 mg, 0.178 mmol) was treated with \(N\)-methyl-4-methoxyaniline 241 (73 mg, 0.533 mmol), 20 mL of isopropanol, and 2 drops of conc. HCl in a 100 mL round bottom flask. The reaction mixture was heated to reflux for 20 hours. The solvent was evaporated and neutralized with 4 mL of ammonia (7N) in methanol. To the solution was added silica gel, four times the weight of the reaction mixture, and the solvent was removed under reduced pressure to provide a plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with 1\% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to give a solid which was further deprotected using 4 mL 1N NaOH solution in 30 mL isopropanol upon reflux for 4 hours. The solvent was evaporated to obtain a brown colored solid. The resulting precipitate was then dissolved in methanol. To the solution was added silica gel, four times the weight of the reaction mixture, and the solvent was removed under reduced pressure to provide a plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with 0.5\% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to provide 183 in 60\% yield. TLC \(R_f = 0.42\) (CHCl\(_3\)/MeOH, 10:1); mp 180.1–180.6 °C (lit.\(^{182}\) 179.0–180.0 °C). \(^1\)H NMR (DMSO-\(d_6\)): \(\delta = 3.30\) (s, 3H, OCH\(_3\)), 3.66 (s, 3H, N-CH\(_3\)), 6.55 (s, 2H, NH\(_2\), exch), 6.76–7.25 (m, 7H, Ar), 11.65 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C\(_{18}\)H\(_{16}\)ClN\(_5\)O: C, 61.10; H, 4.56; N, 19.79; Cl, 10.02. Found: C, 60.91; H, 4.63; N, 19.74; Cl, 10.19.
*N*-**(4-Methoxyphenyl)**-*N*-**methyl-9H-pyrimido[4,5-b]indole-2,4-diamine (185).*

Compound 234 (60 mg, 0.178 mmol) was treated with *N*-methyl-4-methoxyaniline 241 (73 mg, 0.533 mmol), 20 mL of *n*-butanol, and 2 drops of conc. HCl in a 100 mL round bottom flask. The reaction mixture was heated to reflux for 76 hours. The solvent was evaporated and neutralized with 4 mL of ammonia (7N) in methanol. To the solution was added silica gel, four times the weight of the reaction mixture, and the solvent was removed under reduced pressure to provide a plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with 1% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to give a solid which was further deprotected using 4 mL 1N NaOH solution in 10 mL isopropanol upon reflux for 2 hours. The solvent was evaporated to obtain a brown colored solid. The resulting precipitate was then dissolved in methanol. To the solution was added silica gel, four times the weight of the reaction mixture, and the solvent was removed under reduced pressure to provide a plug. The plug was transferred on top of a column packed with silica gel, twenty times the weight of plug, eluted with 0.5% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to provide 185 in 62% yield. TLC *R* <sub>f</sub> = 0.70 (CHCl<sub>3</sub>/MeOH, 5:1); mp 246.8–247.2 °C (lit. 178 245.3–245.7 °C). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ = 3.34 (s, 3H, OCH<sub>3</sub>), 3.73 (s, 3H, N-CH<sub>3</sub>), 5.77–5.79 (m, 1H, Ar), 6.21 (s, 2H, NH<sub>2</sub>, exch), 6.53–6.57 (m, 1H, Ar), 6.90–6.99 (m, 3H, Ar), 7.14–7.17 (m, 3H, Ar), 11.24 (s, 1H, 9-NH, exch). Elemental analysis calculated (%) for C<sub>18</sub>H<sub>17</sub>N<sub>5</sub>O·0.20CH<sub>3</sub>OH: C, 67.05; H, 5.52; N, 21.46. Found: C, 66.97; H, 5.47; N, 21.55.
**Ethyl 2-amino-4-bromo-1H-indole-3-carboxylate (252).**

To an ice-cold solution of ethyl cyanoacetate (5.41 g, 57.8 mmol) in anhydrous DMF (170 mL) under argon atmosphere was added sodium hydride (1.15 g, 47.8 mmol). Thus formed white suspension was stirred for 15 min and then treated with 1,2-dibromo-3-nitrobenzene 192 (4 g, 14.2 mmol). The suspension was heated at 100 °C for 3 h. After cooling the reaction mixture to room temperature, 50 mL H2O was added and the resulting mixture was acidified to pH 2.0 with conc. HCl. The mixture was extracted with ether (3x) and then the combined organic phases were dried using anhydrous sodium sulfate and concentrated to give ethyl (2-bromo-6-nitrophenyl)(cyano)acetate 251 as a yellow oil. The material was used directly for the next step.

A solution of 251 (3.42 g, 11 mmol) in glacial AcOH (150 mL) was treated with a single charge of zinc dust (10.26 g, 156 mmol). The mixture was heated at 60 °C for 45 min and then recharged with Zn dust (3.42 g, 52 mmol). After heating for another 105 min, the mixture was cooled to room temperature and then filtered through a pad of Celite. The pad was washed well with AcOH, the filtrate was concentrated and then neutralized with 5% aq. NaHCO3. The precipitate was collected and then dissolved in methanol. Silica gel (5 g) was added to make the plug and was then purified by column chromatography, eluting sequentially with 0% and 1% MeOH in CHCl3. The fractions containing the pure product (TLC) were pooled and evaporated to give 252 (55% from 192) as a brown solid. TLC Rf = 0.30 (CHCl3/MeOH, 10:1 with 2 drops of conc. NH4OH); mp 136.1–137.0 °C.

1H NMR (400 MHz, DMSO-d6): δ = 1.27–1.31 (t, 3H, CH3); 4.17–4.22 (q, 2H, CH2); 6.75–6.79 (m, 1H, Ar); 6.82 (bs, 2H, NH2, exch); 7.11–7.14 (m, 2H, Ar); 10.93 (s, 1H, NH, exch). 1H NMR agreed well with the literature reported values.
2-Amino-5-bromo-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (253).

A mixture of ethyl 4-bromo-2-amino-1H-indole-3-carboxylate 252 (400mg, 1.41 mmol), carboximidic chloride hydrochloride (325 mg, 2.82 mmol) and methyl sulfoxide (3 g) was stirred at 120 °C for 14 h. The reaction mixture was neutralized by ammonia in methanol (2–3 mL) and then 40 mL methanol and 3 g silica gel were added. Thus obtained plug was purified by column chromatography on silica gel sequentially eluting with 1%, 5% and 10% MeOH in CHCl3 to afford 253 (45%) as a brown solid. TLC $R_f = 0.43$ (CHCl3/MeOH, 5:1 with 2 drops of conc. NH4OH); mp >250 °C; $^1$H NMR (400 MHz, DMSO-$_d_6$) $\delta = 6.59$ (bs, 2H, NH$_2$, exch); 6.97–7.01 (m, 1H, Ar); 7.23–7.25 (m, 2H, Ar); 10.38 (s, 1H, NH, exch); 11.66 (s, 1H, NH, exch). $^1$H NMR agreed well with the literature reported$^{209}$ values.

2-Amino-5-(1-naphthylsulfanyl)-3,9-dihydro-4H-pyrimido[4,5-b]indol-4-one (4g).

Compound 253 (100 mg, 0.36 mmol), 1-naphthylthiol 247 (230 mg, 1.43 mmol), copper iodide (272 mg, 1.43 mmol) and potassium carbonate (396 mg, 2.86 mmol) were added to a Biotage® microwave vial. Around 10 mL DMF was added as solvent and the tube was sealed and purged with argon for 5 min. The reaction was run in a microwave at 180 °C for 20 h. After cooling to room temperature, the DMF was removed under reduced pressure and the crude product was purified by column chromatography, sequentially eluting with 0%, 5% and 8% methanol in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford the desired product 4g as a white solid: yield 24%. TLC $R_f = 0.46$ (CHCl3/MeOH, 5:1 with two drops conc. NH4OH); mp >300 °C (lit.$^{193}$ >250 °C); $^1$H NMR (400 MHz, DMSO-$_d_6$): $\delta = 6.04$ (d, 1H, $J = 8.0$ Hz, C7-CH),
6.54 (bs, 2H, NH₂, exch), 6.75 (t, 1H, J = 7.6 Hz, C6-CH), 6.97 (d, 1H, J = 8.0 Hz, C8-CH), 7.48–7.57 (m, 3H, Ar), 7.73–7.75 (m, 1H, Ar), 7.99–8.02 (m, 2H, Ar), 8.19–8.21 (m, 1H, Ar), 10.40 (s, 1H, 3-NH, exch), 11.53 (s, 1H, 9-NH, exch). HRMS (ESI): m/z calculated for C₂₀H₁₄N₄OS + H⁺ [M+H⁺]: 359.0967. Found: 359.0981. Elemental analysis calculated (%) for C₂₀H₁₄N₄OS·0.14CHCl₃: C, 64.48; H, 3.80; N, 14.94; S, 8.55. Found: C, 64.27; H, 3.69; N, 14.94; S, 8.79. ¹H NMR agreed well with the literature reported values.