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Antiangiogenic and Antitumor Agents: Design, Synthesis, and Evaluation of Novel 2-Amino-4-(3-bromoanilino)-6-benzylsubstituted Pyrrolo[2,3-*d*]pyrimidines as Inhibitors of Receptor Tyrosine Kinases

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Abstract—Several different classes of growth factor receptors containing tyrosine kinases (RTK) are directly or indirectly involved in angiogenesis. Inhibition of these RTKs has provided a new paradigm in the treatment of tumors by restricting their growth and metastasis. We have designed, synthesized and evaluated eleven novel 2-amino-4-(3-bromoanilino)-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines as the first in a series of RTK inhibitors. These analogues were synthesized from appropriate α-bromomethylbenzyl ketones by cyclocondensation with 2,6-diamino-4-pyrimidone to afford the 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines. Chlorination of the 4-position followed by displacement with 3-bromoaniline afforded the target compounds. In some instances, the 2-amino moiety of the pyrrolo[2,3-*d*]pyrimidines was protected prior to the chlorination and displacement followed by deprotection. The compounds were evaluated as inhibitors of vascular endothelial growth factor receptors VEGFR-2 (Flk-1, KDR) and VEGFR-1 (Flt-1); epidermal growth factor receptor (EGFR); and platelet-derived growth factor receptor-β (PDGFR-β). Selected compounds were also evaluated against the growth of A431 cells (which overexpress EGFR) in culture and as inhibitors of angiogenesis in the chicken embryo chorioallantonic membrane (CAM) assay. In each evaluation, a known standard compound was used as a comparison. Of the 11 analogues, five were more potent or equipotent as compared to standard compounds against the growth factor receptors. Two analogues showed superior inhibition of A431 cells in culture compared to the standard compounds. Three analogues were equipotent with the standard compound in the CAM assay and four of the analogues were dual inhibitors of RTKs. The structure–activity relationship for inhibition of different RTKs was quite distinct and different, and for VEGFR-2 and EGFR diametrically opposite. The inhibitory data against the RTKs in this study demonstrates that variation of the substituent(s) in the benzyl ring of these 2-amino-4-anilino 6-benzyl pyrrolo[2,3-*d*]pyrimidines does indeed control both the potency and specificity of inhibitory activity against RTKs.
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Introduction

Angiogenesis is the formation of new blood vessels from existing vasculature.[1] Angiogenesis occurs during development and in normal adults during wound healing, pregnancy and corpus luteum formation. Human disease states associated with angiogenesis include retinopathies, endometriosis, psoriasis, atherosclerosis, rheumatoid arthritis and the growth and metastasis of tumors.[2] Angiogenesis plays a pivotal role in the growth of solid tumors and their invasion and metastasis.[3,4] Thus angiogenesis and metastasis contribute to the poor prognosis seen in patients with highly angiogenic tumors.[5] Inhibition of tumor angiogenesis has thus provided an attractive target for the development of antiangiogenesis agents as antitumor agents. Anti-angiogenic therapy is targeted to non-tumor cells (endothelial cells) which are expected to have less ability to mutate in order to produce resistance compared with tumor cells. Thus, antiangiogenesis agents have afforded new paradigms for the treatment of cancer.[6,7]

Angiogenesis requires the transduction of signals from the extracellular domain of endothelial cells to the nucleus which are, either directly or indirectly, receptor mediated and some of the receptors are receptor tyrosine

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kinases (RTK). RTKs are transmembrane receptors consisting of an extracellular ligand binding domain, a hydrophobic transmembrane domain and a cytoplasmic domain which contains regulatory regions and the catalytic tyrosine kinase domain with binding sites for both ATP and substrate which allows for autophosphorylation and is the critical step in signal transduction pathways. RTK activation often requires the formation of homodimers or heterodimers with other RTKs. Non-receptor tyrosine kinases do not have an extracellular domain and are usually dimers.[7,8]

RTKs consist of families of growth factor receptors such as the platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and epidermal growth factor receptor (EGFR) among several others. Several small molecule inhibitors of RTKs are currently in clinical trials as antitumor agents and the majority of these are targeted at the ATP binding site of tyrosine kinases.[9,10] Some examples of these compounds, along with preclinical compounds, are presented in Figure 1.

EGFR is overexpressed in several tumors.[11–23] Thus, inhibition of EGFR is a worthwhile goal for cancer therapy and several inhibitor of EGFR such as ZD1839 (Iressa) and OS1774 (Tarceva) have completed clinical trials as antitumor agents. ZD1839 has recently been approved by the FDA for non-small cell lung cancer.

VEGFR-2 (Flk-1/KDR) is the principal receptor that mediates VEGF stimulation in angiogenesis. The receptors for VEGF are almost exclusively expressed on endothelial cells. Targeted inhibition or disruption of VEGFR-2 results in abrogation of angiogenesis and tumor growth.[24,25] In addition, VEGF and VEGFRs are overexpressed in many tumor types.[26–28] Several inhibitors of VEGFR-2 have provided antitumor activity. Notable among these are the pyrroloindolinones and quinazolines exemplified by SU5416 (Fig. 1) which has been in clinical trials and ZD6474 which is currently in clinical trials[29–33] as an antitumor agent.

PDGFR-α, PDGFR-β and PDGF have been implicated, indirectly, in inducing VEGF secretion and hence in angiogenesis.[34] Inhibition of PDGFR mediated signaling has direct and indirect effects on tumor growth and metastasis.[35–37] Over expression of PDGFR has been observed in glioblastomas.[23,38,39] ST1-571 (Gleevec) is a potent inhibitor of PDGFR and a v-Abl inhibitor. It has shown excellent anti-leukemia activity in the clinic, has been approved for the treatment of chronic myeloid leukemia and metastatic malignant gastrointestinal stromal tumors, and is under investigation in the treatment of glioblastomas which exploits its PDGFR inhibitory activity.[40]

Recent reports[41] suggest that inhibition of VEGFR-1 (Flt-1) could be a therapeutic target not only for tumor angiogenesis but also for the inflammation associated with tumors. Thus VEGFR-1 is also a viable target against cancer.

Single RTK inhibition by small molecules is a possible mechanism of cancer therapy. Simultaneous targeting of two or more RTKs represents a novel approach for (antiangiogenic) therapy of tumors. These RTKs are present on endothelial cells (VEGFR, PDGFR) in tumor cells (FGFR, PDGFR) and pericytes (FGFR, PDGFR) thus simultaneous inhibition of more than one RTK could provide synergistic effects against tumors.[42–44]

The ATP-binding site of RTKs has been shown to be a viable target for rational drug design.[10] Of these, the most successful have been those based on the quinazoline and indoline scaffolds which are ATP-competitive (Fig. 1). Excellent reviews for quinazolines;[45] pyrido[*d*]pyrimidines;[46,47] and general reviews of tyrosine kinase inhibitors[9,10] in cancer treatment have been published.

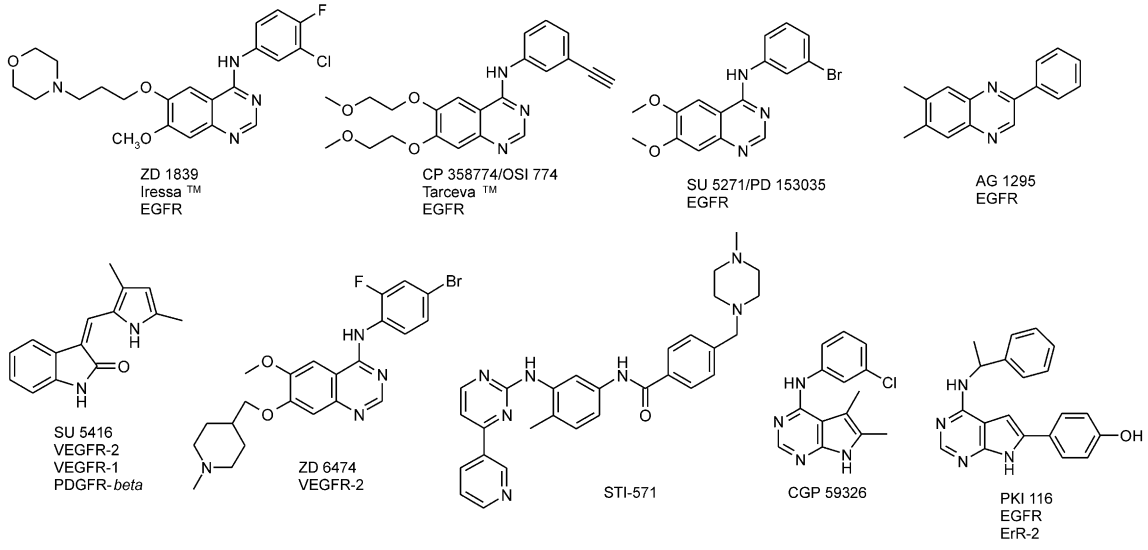


Figure 1. RTK inhibitors.

Molecular modelling

Several crystal structures of RTKs have been published. However, crystal structures of VEGFR2 or PDGFR with ATP or with quinazoline or pyrrolo[2,3-*d*]pyrimidine inhibitors are unknown. After this study was completed a report of the crystal structure of EFGR with quinazoline inhibitors was recently published.[48] Using available X-ray crystal structures of c-AMP dependent protein kinase and FGFR, a general pharmacophore model of the ATP and ATP-competitive inhibitor binding site of RTKs has been proposed[49] and refined.[10]

The model proposed consists of an Adenine region which is a hydrophobic binding site for the adenine ring of ATP as well as for the heterocyclic scaffold of inhibitors such as quinazolines and pyridopyrimidines. The N1- and N6-amino nitrogens of the adenine ring of ATP are hydrogen bonded with a donor acceptor system with two amino acid residues of the Hinge region. For EGFR these residues are Gln 767 and Met 769.[10] For the structure based design of RTK inhibitors, we carried out a sequence homology alignment using SYBYL 6.7 and its Align Structure Using Homology option of the known crystal structures of the insulin receptor kinase (IRK) domain[50] with bound ATP and the unoccupied or apo X-ray crystal structure of VEGFR-2 (Flk1, KDR)[51] (and the inhibitor bound FGFR X-ray crystal structure),[52] followed by the Fit option to further align the Hinge region. For VEGFR-2 the corresponding Hinge region residues are Glu 917 and Cys 919 (Fig. 2). These are important binding sites for ATP and ATP-competitive inhibitors and serve to anchor the heterocyclic portion of the molecule and appropriately orient the other parts of the molecule in the ATP binding site. This modeling alignment, with ATP from IRK modeled into VEGF-2 is shown in Figure 2 and is based on the proposed general pharmacophore for RTKs.[10,49]

An important additional binding site in the Hinge region which involves a hydrogen-bonding backbone carbonyl that would be bonded to a 2-amino group on the pyrimidine ring of an inhibitor of a quinazoline, pyrido[2,3-*d*]pyrimidines or pyrrolo[2,3-*d*]pyrimidine scaffold is shown as the carbonyl of Cys919 in the model in Figure 2. ATP, which lacks this 2-NH_2 moiety, does not use this site. For EGFR this residue corresponds to Met 769 and for VEGFR2, Cys 919. None of the RTK inhibitors in the literature, which contain a pyrrolopyrimidine ring, has a 2-NH_2 group to exploit this H-bonding site. A 2-NH_2 pyrazolopyrimidine was reported by Traxler et al.,[53] however the authors do not ascribe a binding for the 2-NH_2 moiety. We have utilized this 2-NH_2 group as an important integral part of the design of pyrrolo[2,3-*d*]pyrimidine analogues as RTK inhibitors to provide additional binding.

The ATP binding site also contains a Hydrophobic region I (Fig. 2). This pocket extends in the direction of the lone pair of the N7 of ATP and is not used by ATP for binding. This pocket accommodates the phenyl or heterocyclic ring of inhibitor molecules attached to the 4-NH_2 of the pyrimidine ring of quinazolines, pyridopyrimidines or pyrrolopyrimidines. Thus, in Figure 1, the 2-Cl, 4-F-phenyl ring of ZD1839 is accommodated in this region. The size of this pocket varies in different RTKs and is an area that could be exploited for selectivity. Thus several substituted phenyl rings and

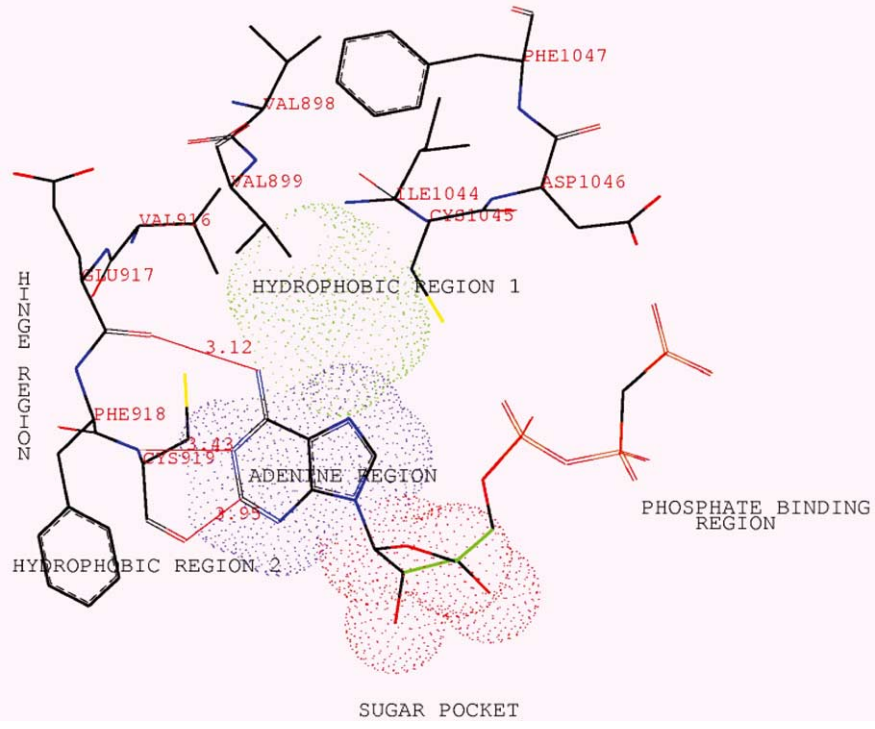


Figure 2. ATP from IRK modeled into VEGFR-2 using SYBYL 6.7.

heterocyclic rings have been utilized on a variety of pyrimidine containing scaffolds to afford potent, and in some cases specific and multiple inhibitors of various RTKs.[9,10,45–47,54–56]

The Sugar pocket (Fig. 2) in the ATP binding site accommodates the sugar moiety of ATP and is hydrophilic but not entirely so. The mode of binding of the 4-anilinophenyl quinazoline based RTK inhibitors, shown in Figure 1, are proposed similar to the binding of ATP. Alternate modes of binding of quinazolines such as Tarceva have recently been reported.[48] For the 6-5 pyrrolo[2,3-*d*]pyrimidines such as CGP59326 (Fig. 1) two different modes of binding are also possible. The first is where the pyrrolo[2,3-*d*]pyrimidine binds like ATP and the quinazolines, where the *m*-Cl-phenyl ring binds in Hydrophobic region I is termed Mode 1 (Fig. 3). An alternate mode of binding proposed by the Novartis group,[10] has the *m*-Cl-phenyl moiety binding in the Sugar pocket. In this mode, the 7-position pyrrolo NH of CGP59326 forms one of the bidentate H-bonds with the Hinge region, substituting for the 4-NH of ATP or quinazolines; the N1 nitrogen of CGP59326 forms the second bidentate H-bond and mimics the N3-nitrogen of quinazoline, this is termed Mode 2. These are illustrated, without the RTK in Figure 3.

In 1994, we[57] proposed similar dual binding modes for 6-5 fused ring furo[2,3-*d*]pyrimidines and pyrrolo[2,3-*d*]pyrimidines for inhibitors of folate related enzymes. Thus Mode 2 is obtained from Mode 1 by rotating 180° around the C_2–H bond. In the absence of X-ray crystal structures of any pyrrolo[2,3-*d*]pyrimidines with any RTK in the literature, the exact mode(s) of binding of these analogues are not known and it is possible that different RTKs may bind the pyrrolo[2,3-*d*]pyrimidine molecules in different or both Modes 1 and 2 shown below.

The Phosphate binding region (Fig. 2) of the RTK model is exposed to solvent and can be exploited to impart water solubility to the inhibitors.

On the basis of the general pharmacophore model,[10,49] adapted to VEGFR-2, presented in Figure 2, we embarked on the design of RTK inhibitors using the pyrrolo[2,3-*d*]pyrimidine scaffold with a 2-NH_2 moiety. To our knowledge this 2-NH_2 substitution has not been explored in the literature to any significant extent. The rationale for including the 2-NH_2 group in our compounds was that it provides a third H-bonding moiety in the Hinge region of RTKs, as described above, and was anticipated to increase binding and consequently potency. The second moiety included in our design was the 4-anilinophenyl ring with a 3-bromo substitution. This moiety has provided potent inhibition of RTKs and is accommodated in the Hydrophobic region I or the Sugar pocket depending on the mode of attachment (i.e., Mode 1 or 2, Fig. 3) for our proposed pyrrolo[2,3-*d*]pyrimidines. As the first analogues in a series of RTK inhibitors, we elected to keep these two substitutions (i.e., the 2-NH_2 group and the 4-amino-(3-Br-phenyl) constant. The purpose was to determine if variation in the phenyl ring of the 6-benzyl moiety of the pyrrolo[2,3-*d*]pyrimidine scaffold would allow for specificity and/or for multiple inhibition of various RTKs, with the 2-NH_2 and 4-amino-(3-Br)phenyl groups being constant. Thus we designed compounds **1**–**11** which contain an amino moiety at the 2-position, a meta-bromo aniline at the 4-position and a benzyl substitution at the 6-position.

Compounds **1**–**11** were modeled in the VEGFR-2 kinase binding site using the apo X-ray crystal structure[51] and the alignment of the ATP from Figure 2. Figure 4 shows a stereo view of the binding of a low energy conformer (SYBYL 6.7 Minimize and Search Options) of compound **5** superimposed on ATP (not shown) modeled in VEGFR-2. In the Hinge region, the anilino NH makes a H-bond with the backbone carbonyl of Glu 917, the N3 with the NH of Cys 919 and the 2-NH_2 with the carbonyl of Cys 919. The 3-Br-phenyl ring lies in the Hydrophobic region I which consists of Leu 1035 and Cys 1045. The 6-benzyl ring lies in the Sugar Pocket and the ortho-methyl group of the benzyl ring makes hydrophobic contact with Val 848. Since these are models, constructed using homology alignment with known X-ray crystal structures, the exact mode of binding of **1**–**11** cannot be predicted, however this model does provide a general template for the design of RTK (VEGFR-2) inhibitors.

Results and Discussion

The target 2-amino-4-(3-bromoanilino)-6-substituted-pyrrolo[2,3-*d*]pyrimidines **1**–**11** were synthesized via the displacement of the 4-chloro analogues with 3-bromo-aniline **50**.[58] The 4-chloro precursors were in turn synthesized from the corresponding 2-amino-4-oxo-pyrrolo[2,3-*d*]pyrimidines.[59,60]

Though there are several reported methods for the synthesis of the 2-amino-4-oxo-pyrrolo[2,3-*d*]pyrimidines,[61,62] there is no report for the preparation of a

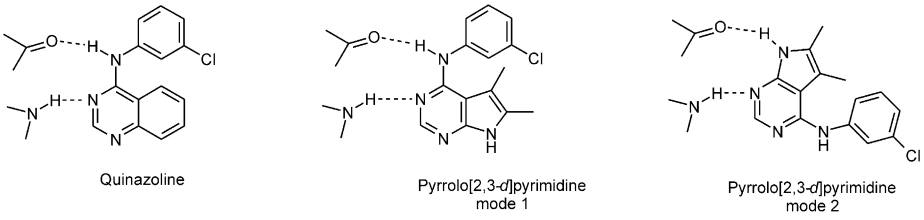


Figure 3.

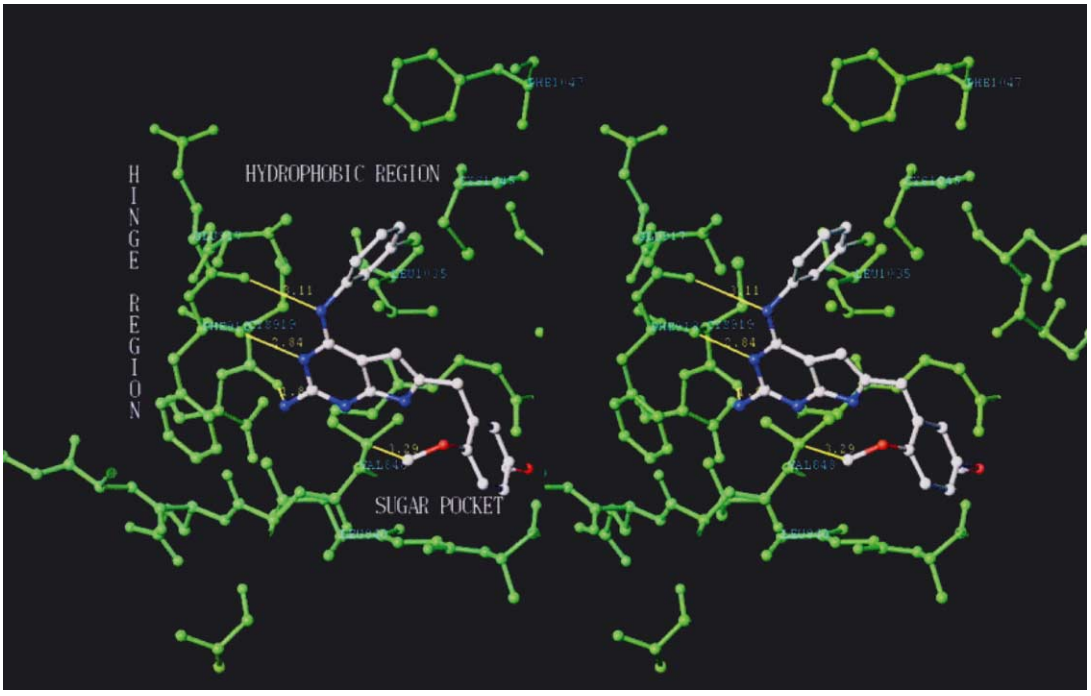
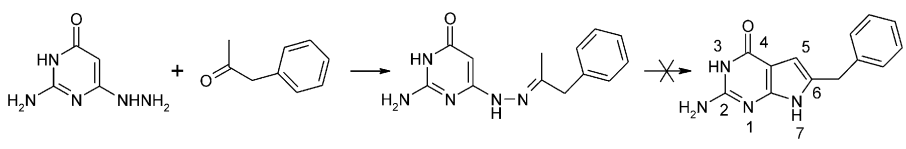


Figure 4. Stereoview: model of compound **5** superimposed on the ATP structure (not shown) in VEGFR-2.

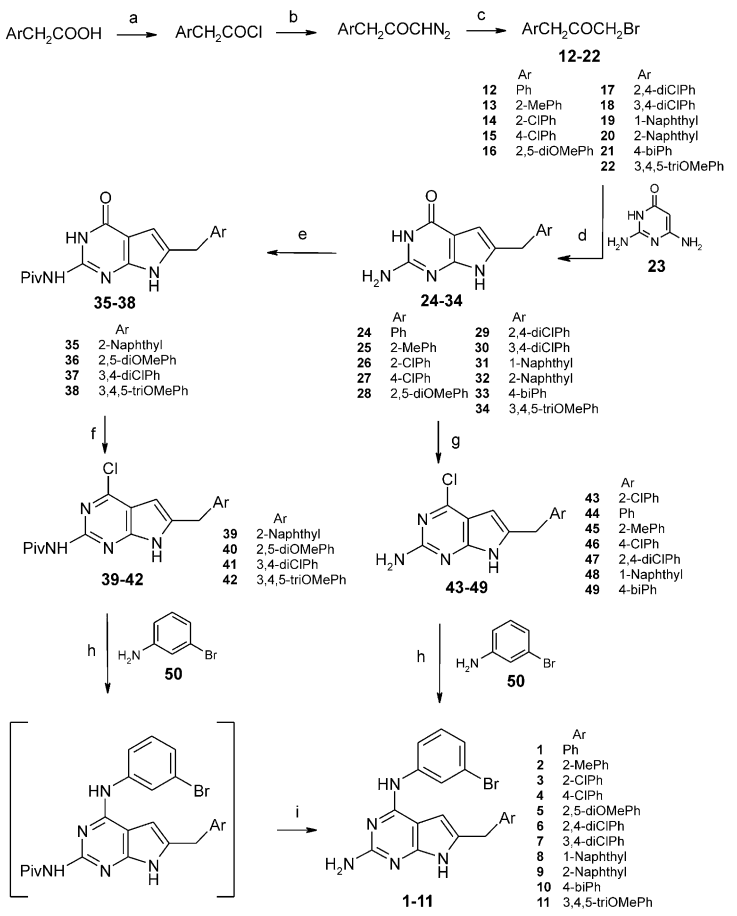
single-carbon bridged side chain at the 6-position. We initially attempted the Fischer indole cyclization (Scheme 1) to form the pyrrolo[2,3-*d*]pyrimidine nucleus using the appropriate methyl benzyl ketone and the hydrazine from 2-amino-4-oxo-6-chloropyrimidine which afforded the hydrazone. Fischer indole cyclization under a variety of neutral, acidic and various temperature conditions did not afford the desired compounds.

The failure of the Fischer indole reaction prompted the search for alternate synthetic methodology. We have reported the cyclocondensation of α-haloketones with substituted pyrimidines to afford a variety of pyrrolo[2,3-*d*]pyrimidines[63] and furo[2,3-*d*]pyrimidines,[57] and envisioned that the α-bromomethyl benzyl ketones with 2,6-diamino-4-oxo-pyrimidine would afford the desired 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidine **24–34**. The precursor α-bromomethyl benzyl ketones **12–22** were obtained from the corresponding substituted phenyl acetic acids or acid chlorides via a three-step sequence (Scheme 2). First the α-diazoketones were formed from the acid chlorides with diazomethane. Treatment of the α-diazoketones with HBr gas was reported to afford the α-bromoketones.[64] In our case, 47.5% aqueous HBr worked just as well to afford the α-bromomethyl benzyl ketones **12–22** and no *O*-demethylated intermediates **16** and **22**, as would be expected from HBr gas, were obtained.

Cylocondensation of the α-bromomethyl benzyl ketones **12–22** with 2,6-diamino-4-oxo-pyrimidine **23** was successful under different solvent (DMF, DMSO, water) and temperature (room temperature to 80 °C) conditions both with and without base (Et_3N, K_2CO_3). The optimized conditions were room temperature in DMF for 3 days which afforded the 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines **24–34** in 45–60% yield. With **24–34** in hand, the conversion of the 4-oxo to the corresponding 4-chloro was attempted with $POCl_3$, $SOCl_2$, or $PCl_5/POCl_3$. None of these reagents afforded the desired product, instead decomposition of the starting material was observed. Clearly the 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines **24–34** were sensitive to the strong acidic conditions. Thus the chlorination was attempted with $POCl_3$ and a base $PhNMe_2$. The 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines **24–27**, **29**, **31** and **33** in $POCl_3$ with $PhNMe_2$ at reflux afforded 2-amino-4-chloro-pyrrolo[2,3-*d*]pyrimidines **43–49** in 25–45% yield. The low yield of this step was probably, in part, due to the absence of drying the $PhNMe_2$ and distillation of $POCl_3$ prior to use as suggested in the literature.[60] However, protection of the 2-amino groups of **28**, **30**, **32** and **34** with the pivaloyl group followed by reflux with $POCl_3$ afforded a smooth conversion to the 2-pivaloylamino-4-chloro-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidines **39–42** in 75–81% yield. [59]



Scheme 1.



Scheme 2. (a) $SOCl_2$, benzene, reflux, 1 h; (b) CH_2N_2/Et_2O, 30 min; (c) 47.5% HBr, 70–80 °C, 1 h; (d) DMF, rt, 3 days; (e) Piv_2O, DMF, DMAP, Et_3N, 60–70 °C, 2 days; (f) $POCl_3$; (g) $POCl_3$, $PhNMe_2$ (h) *i*-PrOH, 2–3 drops of concd HCl, reflux, 30 min, (i) 15% KOH, 1,4-dioxane, reflux, 10 h.

The displacement of the 4-chloro moiety of **39–42** or **43–49** with 3-bromo aniline **50** to give the target compounds **1–11** occurred as reported in the literature.[58] The reaction condition requires 2–3 drops of concd HCl without which no reaction occurred, even at reflux for 24 h. Compared with the unprotected compounds **43–49** where the yields of **1**, **2**, **3**, **4**, **6**, **8** and **10** were 70–80%, the reaction of the 2-amino protected compounds **39–42** occurred faster and afforded a quantitative yield of the pivaloyl protected target compounds. However, deprotection of the 2-pivaloyl group did not occur under mild basic conditions, such as 1 N NaOH in THF or methanol at room temperature or high temperature (100 °C). Stronger conditions of reflux in 1,4-dioxane with 15% KOH for 10 h was required for deprotection to afford **5**, **7**, **9**, and **11**.

Kinase activity of the compounds were evaluated using human tumor cells known to express high levels of EGFR, Flk1, Flt1, PDFGRβ, and FGFR1 using a phosphotyrosine ELISA.[65,66] Compounds known to inhibit the particular RTKs were used as positive controls for these assays. The effect of compounds on cell proliferation was measured using A431 cancer cells, shown to overexpress EGFR, because EGFR has been shown to play a role in the overall survival of the cells.[67] Cell proliferation was assessed using CYQUANT®, a DNA intercalating dye that has been shown to give a linear approximation of cell number.[68] Finally, the effect of selected compounds on blood vessel formation was assessed using the chicken embryo chorioallantoic membrane (CAM) assay, a standard test for angiogenesis.[69] In this assay, purified angiogenic growth factors are placed locally on a vascularized membrane of a developing chicken embryo together with possible inhibitors. Digitized images of the vasculature are taken at 48 h after growth factor administration and the number of vessels per unit area evaluated as a measure of vascular density.

Since the IC_{50} values of compounds vary under different assay conditions, we used a standard (control) compound in each of the evaluations. For VEGFR-2 the standard was SU5416; for VEGFR-1 the standard was (4-chloro-2-fluoro-phenyl)-(6,7-dimethoxy-quinazolin-4-yl)-amine; for EGFR the standard was PD153035; for PDGFR the standard was AG1295; for the cytotoxicity study against the growth of A431 cells in culture the standard was PD153035. Cisplatin and SU5416 were also used along with the other standard analogue in the A431 assay. SU5416 was also used as the standard for the antiangiogenic CAM assay.

In the VEGFR-2 assay (Table 1), the 2-CH_3 and the 2,5-diOMe substituted compounds **2** and **5** were the most potent and were 4- and 8-fold more potent,

Table 1. IC_{50} values (μM) of kinase inhibition, A431 cytotoxicity and inhibition of the CAM assay

Compd	VEGFR-2 inhibition	EGFR inhibition	A431 cytotoxicity	PDGFR-β inhibition	VEGFR-1 inhibition	CAM angiogenesis inhibition
1	>50	1.67±0.3 (2)	31.8±6.3 (2)	>50	>50	
2	0.25±0.04 (2)[a]	9.19±1.8 (2)	1.21±0.42 (2)	>50	>50	1.21±0.23 (2)
3	5.58±0.69 (2)	4.31±1.75 (2)	>50	>50	26.8±4.1(2)	1.70±0.34 (3)
4	8.28±0.53 (2)	17.42±3.9 (2)	28.6±5.1 (2)	>50	42.7±6.1 (2)	<0.1 (toxic)
5	0.62±0.21 (3)	12.62±3.3 (2)	>50	8.92±1.6 (2)	31.1±5.8 (2)	1.32±0.42 (2)
6	28.11±9.9 (2)	0.23±0.06 (2)	2.8±1.1 (3)	17.0±5.6 (2)	>50	10.8±3.2 (2)
7	>50	19.77±5.6 (2)	33.5±6.2 (2)	>50	>50	
8	5.08±0.83 (2)	>50	>50	>50	19.2±4.3 (2)	0.053±0.007 (2)
9	>50	1.24±0.21 (2)	33.2±5.9 (2)	>50	15.2±2.9 (2)	
10	5.97±0.78 (2)	6.16±1.2 (2)	23.5±5.2 (2)	>50	>50	0.052±0.008 (2)
11	9.42±1.9 (2)	>50	42.1±18.5 (2)	14.7±3.4 (2)	>50	0.43±0.03 (2)
SU5416	2.43±0.32 (15)		19.2±4.2 (5)			0.032±0.005 (13)
PD153035		0.2 4±0.042 (16)	12.6±2.9 (6)			
PD168393			12.6±4.8 (2)			
AG1295				6.2±1.6 (7)		
VEGFRkinase Inhibitor					17.7±2.3 (8)	

[a]Number in parentheses is *n*.

respectively, than the standard, SU5416. The 1-naphthyl analogue **8**, the 2-Cl analogue **3** and the 4-biphenyl analogue **10** were equipotent and were about one half as potent as SU5416. The other analogues (**11**, **7** and **4**) were 3- to 12-fold less potent than SU5416 or inactive (**1**, **7** and **9**). These results indicate that a 2-substitution of a Me or OMe on the phenyl ring afford the most potent analogues. The 1-naphthyl, also a 2-substituted phenyl, is reasonably potent as is the 2-Cl analogue. Thus *ortho* or 2-substitutions are conducive to potent VEGFR-2 inhibitory activity and electron donating groups are better than electron withdrawing groups (**2** vs **3**). Both the electronic nature and the location of the substituents on the phenyl ring were important for VEGFR-2 inhibitory activity. A comparison of the 1-naphthyl and 2-naphthyl analogues **8** and **9** reveals a more than 10-fold difference in activity indicating that a 2,3-disubstituted phenyl is much better than a 3,4-disubstituted phenyl. In addition, the decrease in inhibitory activity of the 2-naphthyl vs the 1-naphthyl may also indicates a subtle distinction of bulk tolerance at the VEGFR-2 kinase domain.

Against EGFR the most potent compound was the 2,4-diCl analogue **6** and it was equipotent with the standard EGFR inhibitor, PD153035. The other analogues had comparatively lower inhibitory effects and ranged from 6- to 100-fold less potent than PD153035. Removal of either of the Cl moieties of **6** to afford **3** or **4** leads to a considerable loss in inhibitory activity as does moving the 2-Cl to the 3-position (compound **7**). Remarkably, the 2-naphthyl analogue (also a 3,4-disubstituted phenyl) was significantly more potent (16.5-fold) than **7**. In addition, comparison of the 1- and 2-naphthyl analogues **8** and **9** provides a greater than 42-fold difference in activity indicating that, in contrast to VEGFR-2, for EGFR a 2-naphthyl group is much better than a 1-naphthyl and again may reflect subtle differences in bulk tolerance at the EGFR kinase domain. Thus, for EGFR a 2,4-disubstituted phenyl with electron withdrawing groups provided the most potent analogues and was in contrast to VEGFR-2 where the most potent analogues were mono ortho substituted or 2,5-disubstituted analogues with electron donating groups.

Cytotoxicity studies against the growth of A431 (human cancer cells that overexpress EGFR) in culture provided interesting results. Two of the standard compounds SU5416, and PD153035 along with cisplatin were compared with analogues **1**–**11**. The most potent analogue was the 2-Me compound **2**. This was 16-, 10.5- and 7-fold more potent than SU5416, PD153035 and cisplatin, respectively. Though this analogue was not the most potent against EGFR. The most potent analogue against EGFR, compound **6**, was the second most active analogue against A431 cells, and was 7-, 4.5- and 3-fold more potent than the standard compounds SU5416, PD153035 and cisplatin, respectively. All of the other analogues were less potent than the standard compounds. The data for most of the analogues do correlate EGFR inhibitory activity (or inactivity) with A431 inhibitory activity (or inactivity). However, there is no relative correlation for **1**, **3** and **9**. This could reflect transport and/or other factors which influence the cytotoxic activity against cells in culture. Thus two analogues, **2** and **6**, were remarkably potent against the growth of A431 cells in culture and were from 3- to 16-fold more potent than the standard compounds.

Against PDGFRβ compounds **5**, **6**, and **11** were similar in activity to the standard analogue AG1295 with activities of 1.5-, 3- and 2-fold less potent, respectively, than AG1295. All of the monosubstituted analogues, the unsubstituted analogue and the 1- and 2-naphthyl analogues were essentially inactive. This suggests that for PDGFRβ inhibition a di- or trisubstituted phenyl ring is necessary and that electron donating groups were slightly better than electron withdrawing groups.

The involvement of Flt1 (VEGFR-1) in angiogenesis has not been clearly delineated; however, recent reports[41] suggest its involvement in tumor angiogenesis and inflammation associated with tumors.[70–72] Thus we also evaluated compounds **1**–**11** as inhibitors of

VEGFR-1. The results are indicated in Table 1. The bulky 1- and 2-naphthyl analogues **8** and **9** were equipotent with the standard VEGFR-1 inhibitor 4-(4-chloro, 2-fluoro phenyl)amino-6,7-dimethoxyquinazoline and indicates a preference for bulk on the phenyl ring. However, both the biphenyl and triOMe analogues **10** and **11** were inactive against VEGFR-1. Compounds **3**, **4** and **5** were about one half as potent as **8** and **9**. The remaining analogues were inactive.

On the basis of their activity against VEGFR-2 selected compounds were evaluated in the CAM angiogenesis assay. Two compounds, **8** and **10** were equipotent with SU5416 in inhibiting bFGF/VEGF induced blood vessel formation. Compound **11** was the next most potent analogue. The other analogues were much less potent than SU5416. The two most potent VEGFR-2 inhibitors, **2** and **5**, had good activity in the CAM assay but were not the most potent. Compounds **8** and **10** were reasonably potent against VEGFR-2 and were also highly active in the CAM assay.

Multiple inhibitors of RTKs are also of interest in the treatment of cancer.[42–44] On the basis of the data discussed above, compound **5** shows potent inhibition of VEGFR-2 (IC_{50} 0.6 μM) as well as PDGFRβ (IC_{50} 8.9 μM) kinase inhibition and hence is a potent dual inhibitor, with activities which supercede both standard compounds. Compounds **3** and **8** can also be classified as dual inhibitors of VEGFR-1 (Flt) and VEGFR-2 kinases. Compound **9** has both EGFR and VEGFR-1 kinase inhibition inhibitory activity and can also be considered a dual acting agent.

Conclusion

Eleven novel 2-amino-4-anilino 6-methylphenyl substituted pyrrolo[2,3-*d*]pyrimidines **1**–**11** were designed and synthesized as RTK inhibitors. The biological evaluation showed several analogues had remarkable inhibitory activity against VEGFR-2, EGFR, A431 proliferation, PDGFRβ, VEGFR-1 and in the CAM angiogenesis assay. Of the analogues evaluated, five (compounds **1**, **2**, **5**, **6**, and **8**) were much more potent or equipotent as compared to the standard compounds. Two analogues, **2** and **6**, showed potent cytotoxic effects against A431 cells in culture and two analogues, **8** and **10**, demonstrated high antiangiogenic activity in the CAM assay. In addition, four analogues (compounds **3**, **5**, **8**, and **9**) showed dual inhibitory activity. The structure–activity relationship for inhibition of different RTKs was quite distinct and different, and for VEGFR-2 and EGFR diametrically opposite. The inhibitory data against the RTKs in this study demonstrates that variation of the substituent(s) in the benzyl ring of these 2-amino-4-anilino 6-benzyl pyrrolo[2,3-*d*]pyrimidines does indeed control both the potency and specificity of inhibitory activity against RTKs. Using the analogues described in this study as leads, an extensive SAR and structure-based design and synthesis of further RTK inhibitors are currently in progress. In addition, selected compounds from this study are also being evaluated in appropriate animal models for solid tumors.

Experimental

Melting points were determined on a Mel-Temp II melting point apparatus with FLUKE 51 K/J electronic thermometer and were uncorrected. Nuclear magnetic resonance spectra for proton (^1H) were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift values were expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = double, t = triplet, q = quartet, m = multiplet, br = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. High-resolution mass spectra (HRMS), using Electron Impact (EI), were recorded on a VG Autospec (Fisons Instruments) micromass (EBE Geometry) double focusing mass spectrometer. Thin-layer chromatography (TLC) was performed on Polygram Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC were by volume. Column chromatography was performed on 230–400 mesh silica gel purchased from Aldrich Chemical Co., Milwaukee, WI, USA. All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5 at 75–110 °C. Elemental analysis was performed by Altlantic Microlabs, Norcross, GA, USA. Element compositions are within ±0.4% of calculated values. 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 ^1H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received.

General procedure for the synthesis of compounds 24–34

A solution of substituted phenyl acetic acid (5 mmol) in 5 mL of dry benzene and 5 mL of thionyl chloride was refluxed for 1 h and the colorless solution was evaporated in vacuo. The resulting acid chloride was dissolved in 8 mL of ether and added dropwise with stirring to 30–40 mL of ethereal diazomethane (about 13 mmol, made from 20 mmol of nitromethyl urea)[73] at 0–5 °C. The yellow solution was allowed to stand at room temperature for 1 h, then 5 mL of 47.5% aqueous HBr was added dropwise to the solution and the yellow mixture was heated at 70–80 °C (oil bath) for 1 h. The reaction was cooled to room temperature and the ether layer was separated, washed with water (10 mL), saturated $NaHCO_3$ aqueous solution (10 mL) and water (10 mL) and dried (anhydrous Na_2SO_4). The solution was evaporated to afford the α-bromomethyl benzyl ketones **12**–**22**. The compounds **12**–**22** were not purified further and were used directly in the next step.

The α-bromomethyl benzyl ketone **12**–**22** was placed in a 50-mL flask and an equivalent amount of

2,6-diaminopyrimidin-4-one **22** and 5 mL dry DMF were added. The mixture was stirred at room temperature for 3 days. After half or 1 day, the reaction became a brown or dark solution, at the third day of the reaction, a solid could be precipitated out in some cases. To the reaction mixture was added 1 g of silica gel and the solvent was evaporated in vacuo to afford a dry plug. This plug was placed on the top of 45×150 mm silica gel column and eluted with $CHCl_3$/MeOH (a gradient elution, 2% MeOH in $CHCl_3$, and 5% MeOH in $CHCl_3$). Fractions containing the product (TLC) were pooled and evaporated to afford the pure 2-amino-4-oxo-6-substituted benzyl pyrrolo[2,3-*d*]pyrimidine **24–34**.

2-Amino-4-oxo-6-benzyl-pyrrolo[2,3-*d*]pyrimidine (24). From phenyl acetyl chloride (0.77 g, 5 mmol), 1-bromo-3-phenyl-acetone **12** was obtained using the general procedure described above as a light-yellow oil (0.63 g, 59%). TLC R_f 0.71 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 2H, CH_2), 3.93 (s, 2H, CH_2), 7.30 (m, 5H, Ar–H).

Using the general procedure described above, compound **12** (600 mg, 3.75 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one 23 to afford the compound **24** (400 mg, 45%) as a yellow solid. Mp: 270 °C. TLC R_f 0.50 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 2H, CH_2), 5.85 (s, 1H, C5-CH), 5.98 (s, 2H, NH_2), 7.10–7.30 (m, 5H, Ar–H), 10.15 (s, 1H, NH), 10.95 (s, 1H, NH). Anal. ($C_{13}H_{12}N_4O$): C, H, N.

2-Amino-4-oxo-6-(2-methylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (25). From 2-methylphenylacetic acid (0.75 g, 5 mmol), 1-bromo-3-(2-methylphenyl)-acetone **13** was obtained using the general procedure described above as a yellow oil (0.71 g, 62%). TLC R_f 0.58 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 2.25 (s, 3H, CH_3), 3.85 (s, 2H, CH_2), 3.95 (s, 2H, CH_2), 7.10–7.22 (m, 4H, Ar–H).

Using the general procedure described above, compound **13** (700 mg, 3 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **25** (400 mg, 52%) as a yellow solid. Mp: 290 °C. TLC R_f 0.56 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 3.90 (s, 2H, CH2), 5.85 (s, 1H, C5-CH), 6.03 (s, 2H, NH_2), 7.15–7.30 (m, 4H, Ar–H), 10.10 (s, 1H, NH), 10.94 (s, 1H, NH). Anal. ($C_{14}H_{14}N_4O$): C, H, N.

2-Amino-4-oxo-6-(2-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (26). From 2-chlorophenylacetic acid (0.86 g, 5 mmol), 1-bromo-3-(2-chlorophenyl)-acetone **14** was obtained using the general procedure described above as a white solid (0.81 g, 65%). Mp: 38–41 °C. TLC R_f 0.76 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 4.05 (s, 2H, CH_2), 4.10 (s, 2H, CH_2), 7.20–7.50 (m, 4H, Ar–H).

Using the general procedure described above, compound **14** (800 mg, 3.2 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the product **26** (430 mg, 49%) as a yellow solid. Mp: 290 °C. TLC R_f 0.52 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.92 (s, 2H, CH_2), 5.77 (s, 1H, C5-CH), 6.01 (s, 2H, NH_2), 7.25–7.45 (m, 4H, Ar–H), 10.16 (s, 1H, NH), 10.97 (s, 1H, NH). Anal. ($C_{13}H_{11}N_4OCl·0.8H_2O$): C, H, N, Cl.

2-Amino-4-oxo-6-(4-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (27). From 4-chlorophenylacetic acid (0.86 g, 5 mmol), 1-bromo-3-(4-chlorophenyl)-acetone **15** was obtained using the general procedure described above as a yellow oil (0.63 g, 51%). TLC R_f 0.74 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.88 (s, 2H, CH_2), 3.91 (s, 2H, CH_2), 7.31–7.12 (dd, 4H, Ar–H).

Using the general procedure described above, compound **15** (500 mg, 2 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **27** (670 mg, 54%) as a yellow solid. Mp: 255 °C. TLC R_f 0.53 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.81 (s, 2H, CH_2), 5.86 (s, 1H, C5-CH), 6.02 (s, 2H, NH_2), 7.20–7.35 (m, 4H, Ar–H), 10.17 (s, 1H, NH), 10.94 (s, 1H, NH). Anal. ($C_{13}H_{11}N_4OCl$): C, H, N, Cl.

2-Amino-4-oxo-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (28). From 2,5-dimethoxyphenylacetic acid (0.98 g, 5 mmol), 1-bromo-3-(2,5-dimethoxyphenyl)-acetone **16** was obtained using the general procedure described above as a yellow oil (0.52 g, 45%). TLC R_f 0.46 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.75 (s, 6H, 2×OCH_3), 3.80 (s, 2H, CH_2), 4.18 (s, 2H, CH_2), 6.84–6.70 (m, 3H, Ar–H).

Using the general procedure described above, compound **28** (510 mg, 2.2 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **28** (230 mg, 40%) as a yellow solid. Mp: 275 °C. TLC R_f 0.42 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.64 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.73 (s, 2H, CH_2), 5.76 (s, 1H, C5-CH), 5.97 (s, 2H, NH_2), 6.66–6.76 (m, 2H, Ar–H), 6.86–6.89 (m, 1H, Ar–H), 10.13 (s, 1H, NH), 10.85 (s, 1H, NH). Anal. ($C_{15}H_{16}N_4O_3$): C, H, N.

2-Amino-4-oxo-6-(2,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (29). From 2,4-dichlorophenylacetic acid (1.02 g, 5 mmol), 1-bromo-3-(2,4-dichlorophenyl)-acetone **17** was obtained using the general procedure described above as a white solid (0.72 g, 51%). Mp: 70–73 °C. TLC R_f 0.60 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.95 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 7.20–7.45 (m, 3H, Ar–H).

Using the general procedure described above, compound **17** (710 mg, 2.5 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **29** (440 mg, 56%) as a yellow solid. Mp: 265 °C. TLC R_f 0.51 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.94 (s, 2H, CH_2), 5.85 (s, 1H, C5-CH), 7.25–7.60 (m, 3H, Ar–H), 8.24 (s, br., 2H, NH_2), 10.60 (s, 1H, NH), 11.20 (s, 1H, NH). Anal. ($C_{13}H_{10}N_4OCl_2·0.6H_2O$): C, H, N, Cl.

2-Amino-4-oxo-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (30). From 3,4-dichlorophenylacetic acid (1.02 g,

5 mmol), 1-bromo-3-(3,4-dichlorophenyl)-acetone **18** was obtained using the general procedure described above as a white solid (0.64 g, 45%). Mp: 70–72 °C. TLC R_f 0.50 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 2H, CH_2), 3.91 (s, 2H, CH_2), 7.05–7.45 (m, 3H, Ar–H).

Using the general procedure described above, compound **18** (630 mg, 2.2 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **30** (450 mg, 65%) as a yellow solid. Mp: 290 °C. TLC R_f 0.52 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.83 (s, 2H, CH_2), 5.92 (s, 1H, C5-CH), 6.01 (s, 2H, NH_2), 7.21-7.55 (m, 3H, Ar–H), 10.17 (s, 1H, NH), 10.94 (s, 1H, NH). Anal. ($C_{13}H_{10}N_4OCl_2$·0.1CH_3OH): C, H, N, Cl.

2-Amino-4-oxo-6-(1-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (31). From 1-naphthylacetic acid (0.93 g, 5 mmol), 1-bromo-3-(1-naphthyl)-acetone **19** was obtained using the general procedure described above as a white solid (0.65g, 55%). Mp: 53–56 °C. TLC R_f 0.53 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 2H, CH_2), 4.38 (s, 2H, CH_2), 7.55 (m, 4H, Ar–H), 7.90 (m, 3H, Ar–H).

Using the general procedure described above, compound **19** (470 mg, 2.23 mmol) reacted with equivalent 2,6-diaminopyrimidin-4-one **23** to afford the compound **31** (470 mg, 72%) as a yellow solid. Mp: 255 °C. TLC R_f 0.47 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 4.30 (s, 2H, CH_2), 5.75 (s, 1H, C5-CH), 6.05 (s, 2H, NH_2), 7.35–7.55 (m, 4H, Ar–H), 8.80–7.15 (m, 3H, Ar–H), 10.15 (s, 1H, NH), 11.05 (s, 1H, NH). Anal. ($C_{17}H_{14}N_4$·0.4H_2O): C, H, N.

2-Amino-4-oxo-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (32). From 2-naphthylacetic acid (0.93 g, 5 mmol), 1-bromo-3-(2-naphthyl)-acetone **20** was obtained using the general procedure described above as a white solid (0.78 g, 60%). Mp: 80–82.5 °C. TLC R_f 0.65 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 2H, CH_2), 4.10 (s, 2H, CH_2), 7.20–8.80 (m, 7H, Ar–H).

Using the general procedure described above, compound **20** (780 mg, 2.96 mmol) reacted with equivalent 2,6-diaminopyrimidin-4-one **23** to afford the compound **32** (450 mg, 54%) as a yellow solid. Mp: 270 °C. TLC R_f 0.50 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 4.00 (s, 2H, CH_2), 5.90 (s, 1H, C5-CH), 6.05 (s, 2H, NH_2), 7.95–7.50 (m, 7H, Ar–H), 10.20 (s, 1H, NH), 11.00 (s, 1H, NH). Anal. ($C_{17}H_{14}N_4O$·0.8H_2O): C, H, N.

2-Amino-4-oxo-6-(4-phenylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (33). From 4-biphenylacetic acid (1.06 g, 5 mmol), 1-bromo-3-(4-biphenyl)-acetone **21** was obtained using the general procedure described above as white solid (0.85 g, 59%). Mp: 71–73 °C. TLC R_f 0.70 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 2H, CH_2), 4.00 (s, 2H, CH_2), 7.28–7.50 (m, 5H, Ar–H), 7.52–7.65 (dd, 4H, Ar–H).

Using the general procedure described above, compound **21** (800 mg, 2.7 mmol) reacted with equivalent 2,6-diaminopyrimidin-4-one **23** to afford the compound **33** (410 mg, 48%) as a yellow solid. Mp: 275 °C. TLC R_f 0.51 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.86 (s, 2H, CH_2), 5.90 (s, 1H, C5-CH), 6.00 (s, 2H, NH_2), 7.31–7.46 (m, 4H, Ar–H), 7.56–7.64 (m, 5H, Ar–H), 10.16 (s, 1H, NH), 10.96 (s, 1H, NH). Anal. ($C_{19}H_{16}N_4O$·0.1H_2O): C, H, N.

2-Amino-4-oxo-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (34). From 3,4,5-trimethoxy-phenylacetic acid (1.13 g, 5 mmol), 1-bromo-3-(3,4,5-trimethoxyphenyl)-acetone **22** was obtained using the general procedure described above as a white solid (0.66 g, 51%). Mp: 50–53 °C. TLC R_f 0.42 (hexane/EtOAc, 3:1). ^{1}H NMR ($CDCl_3$): δ 3.68 (s, 3H, OCH_3), 3.86 (s, 6H, 2×OCH_3), 3.95 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 7.20–7.45 (s, 2H, Ar–H).

Using the general procedure described above, compound **22** (950 mg, 3.67 mmol) reacted with an equivalent amount of 2,6-diaminopyrimidin-4-one **23** to afford the compound **34** (360 mg, 44%) as a yellow solid. Mp: 235×C. TLC R_f 0.45 ($CHCl_3$/CH_3OH, 5:1). ^{1}H NMR (DMSO-d_6): δ 3.61 (s, 3H, OCH_3), 3.74 (s, 2H, CH_2), 3.77 (s, 6H, 2×OCH_3), 5.90 (s, 1H, C5-CH), 5.98 (s, 2H, NH_2), 6.58 (s, 2H, Ar–H), 10.15 (s, 1H, NH), 10.91 (s, 1H, NH). Anal. ($C_{16}H_{18}N_4O_4$): C, H, N.

General procedure for the synthesis of compounds 35–38

2-Amino-4-oxo-6-substituted-pyrrolo[2,3-*d*]pyrimidine **28**, **30**, **32**, **34** (1–2 mmol), Piv_2O (3 equivalence), DMAP (0.05 equivalence), Et_3N (5 equivalence), and 5 mL of dry DMF were placed in a 50-mL round-bottom flask. The mixture was stirred at 60–70 °C for 2 days, the solid was dissolved completely and formed a dark solution. Then about 500 mg silica gel was added and the solvent was evaporated in vacuo to afford a dry plug. This plug was placed on the top of 15×150 mm silica gel column and eluted with 1% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford pure 2-pivaloylamino-4-oxo-6-substitued-pyrrolo[2,3-*d*]pyrimidine **35–38**.

2-Pivaloylamino-4-oxo-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (35). Compound **35** was synthesized from 2-amino-4-oxo-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine **32** (580 mg, 2.0 mmol) using the general procedure described above to afford **35** 450 mg (60%) as an off yellow solid. Mp: 230 °C, TLC R_f 0.38 ($CHCl_3$/CH_3OH, 10:1). ^{1}H NMR (DMSO-d_6): δ 1.17 (s, 9H, $C(CH_3)_3$), 4.12 (s, 2H, CH_2), 6.12 (s, 1H, C5-CH), 7.41–7.88 (m, 7H, Ar–H), 10.76 (s, 1H, NH), 11.56 (s, 1H, NH), 11.82 (s, 1H, NH). Anal. ($C_{22}H_{22}N_4O_2$·0.3H_2O): C, H, N.

2-Pivaloylamino-4-oxo-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (36). Compound **36** was synthesized from 2-amino-4-oxo-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **28** (300 mg,1.0 mmol) using

the general procedure described above to afford **36** 330 mg (85%) as a yellow solid: Mp: 250 °C. TLC R_f 0.43 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.16 (s, 9H, $C(CH_3)_3$), 3.64 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 3.85 (s, 2H, CH_2), 5.96 (s, 1H, C5-CH), 6.64–6.92 (m, 3H, Ar–H), 10.76 (s, 1H, NH), 11.45 (s, 1H, NH), 11.80 (s, 1H, NH).

2-Pivaloyamino-4-oxo-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (37). Compound **37** was synthesized from 2-amino-4-oxo-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **30** (570 mg,1.8 mmol) using the general procedure described above to afford **37** 300 mg (65%) as a yellow solid. Mp: 250 °C. TLC R_f 0.47 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.16 (s, 9H, $C(CH_3)_3$), 3.97 (s, 2H, CH_2), 6.14 (s, 1H, C5-CH), 7.23–7.58 (m, 3H, Ar–H), 10.75 (s, 1H, NH), 11.51 (s, 1H, NH), 11.83 (s, 1H, NH).

2-Pivaloylamino-4-oxo-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (38). Compound **38** was synthesized from 2-amino-4-oxo-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **34** (400 mg, 1.2 mmol) using the general procedure described above to afford **38** 460 mg (92%) as a yellow solid. Mp: 240 °C. TLC R_f 0.44 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.23 (s, 9H, $C(CH_3)_3$), 3.48 (s, 3H, OCH_3), 3.66 (s, 6H, $2\times OCH_3$), 3.73 (s, 2H, CH_2), 6.60 (s, 1H, C5-CH), 6.78 (s, 2H, Ar–H), 10.73 (s, 1H, NH), 11.50 (s, 1H, NH), 11.80 (s, 1H, NH).

General procedure for the synthesis of compounds 39–42

50-mL round-bottom flask was placed 2-pivaloylamino-4-oxo-6-substitued-pyrrolo[2,3-*d*]pyrimidine **35–38** (about 1 mmol) and $POCl_3$ 5 mL. The mixture was then heated and kept refluxing for 2 h. After evaporation of the excess of $POCl_3$, ice-cold water was added. The reaction mixture was neutralized with $NH_3{\cdot}H_2O$, and extracted with $CHCl_3$ (3×50 mL). The organic phase was combined and dried with Na_2SO_4. Concentration of the chloroform afforded a brown solid that was dissolved in chloroform (2–3 mL) again and was placed to the top of a 15×150 mm column and eluted with 0.1% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford pure 2-pivaloylamino-4-chloro-6-substitued-pyrrolo[2,3-*d*]pyrimidine **39–42**.

2-Pivaloylamino-4-chloro-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (39). Compound **39** was synthesized from 2-pivaloylamino-4-oxo-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine 35 (400 mg, 1.06 mmol) using the general procedure described above to afford 39 350 mg (80%) as an off white solid. Mp: 151–153 °C. TLC R_f 0.83 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.28 (s, 9H, $C(CH_3)_3$), 4.19 (s, 2H, CH_2), 6.20 (s, 1H, C5-CH), 7.20-7.80 (m, 7H, Ar–H), 8.03 (s, 1H, NH), 10.04 (s, 1H, NH). Anal. ($C_{22}H_{21}N_4OCl$): C, H, N, Cl.

2-Pivaloylamino-4-chloro-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (40). Compound **40** was synthesized from 2-pivaloylamino-4-oxo-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **36** (300 mg, 0.86 mmol) using the general procedure described above to afford **40** 270 mg (80%) as an off white solid. Mp: 129–131.5 °C. TLC R_f 0.8 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.20 (s, 9H, $C(CH_3)_3$), 3.65 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.97 (s, 2H, CH_2), 6.01 (s, 1H, C5-CH), 6.78–7.00 (m, 3H, Ar–H), 9.99 (s, 1H, NH), 12.35 (s, 1H, NH).

2-Pivaloylamino-4-chloro-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (41). Compound **41** was synthesized from 2-pivaloylamino-4-oxo-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **37** (280 mg, 0.7 mmol) using the general procedure described above to afford **41** 220 mg (75%) as an off white solid. Mp: 120–123 °C. TLC R_f 0.80 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.29 (s, 9H, $C(CH_3)_3$), 3.95 (s, 2H, CH_2), 6.19 (s, 1H, C5-CH), 6.76–7.23 (m, 3H, Ar–H), 8.06 (s, 1H, NH), 10.84 (s, 1H, NH).

2-Pivaloylamino-4-chloro-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (42). Compound **42** was synthesized from 2-pivaloylamino-4-oxo-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **38** (400 mg, 0.96 mmol) using the general procedure described above to afford 42 340 mg (81%) as an off white solid. Mp: 98–101 °C. TLC R_f 0.75 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 1.21 (s, 9H, $C(CH_3)_3$), 3.61 (s, 3H, OCH_3), 3.75 (s, 6H, $2\times OCH_3$), 3.98 (s, 2H, CH_2), 6.24 (s, 1H, C5-CH), 6.67 (s, 2H, Ar–H), 9.98 (s, 1H, NH), 12.34 (s, 1H, NH).

General procedure for the synthesis of compounds 43–49

2-Amino-4-oxo-6-substitued-pyrrolo[2,3-*d*]pyrimidine **24**, **25**, **26**, **27**, **29**, **31**, and **33** (about 1 mmol), 5 mL of $POCl_3$ and 0.1 mL of $PhNMe_2$ were placed in a 50-mL round-bottom flask. The mixture was heated to reflux and kept stirring for 4 h. After evaporation of the excess of $POCl_3$, ice-cold water was added and the reaction mixture was neutralized with $NH_3{\cdot}H_2O$. The precipitated solid was collected by filtered, and the filtrate was extracted with $CHCl_3$ (3×50 mL). The organic phase was combined and dried (Na_2SO_4). Concentration of the chloroform afforded another solid. Combined these solids and dissolved in methanol, then 500 mg silica gel was added and removed the solvent in vacuo to afford a dry plug. This plug was placed on the top of a 15×150 mm column and eluted with 2% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford pure 2-amino-4-chloro-6-substituted-pyrrolo[2,3-*d*]pyrimidine **43–49**.

2-Amino-4-chloro-6-(2-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (43). Compound **43** was synthesized from 2-amino-4-oxo-6-(2-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **26** (300 mg, 1.1 mmol) using the general procedure described above to afford **43** 140 mg (45%) as a light yellow solid. Mp: 240 °C. TLC R_f 0.50 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.05 (s, 2H, CH_2), 5.77 (s, 1H, C5-CH), 6.45 (s, 2H, NH_2), 7.28–7.46 (m, 4H, Ar–H), 11.51 (s, 1H, NH). Anal. ($C_{13}H_{10}N_4Cl_2{\cdot}0.2H_2O$): C, H, N, Cl.

2-Amino-4-chloro-6-benzyl-pyrrolo[2,3-*d*]pyrimidine (44). Compound **44** was synthesized from 2-amino-4-oxo-6-benzyl-pyrrolo[2,3-*d*]pyrimidine **24** (300 mg, 1.25 mmol) using the general procedure described above to afford **44** 81 mg (25%) as a light yellow solid. Mp: 230 °C. TLC R_f 0.51 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.93 (s, 2H, CH_2), 5.89 (s, 1H, C5-CH), 6.44 (s, 2H, NH_2), 7.31–7.44 (m, 5H, Ar–H), 11.46 (s, 1H, NH).

2-Amino-4-chloro-6-(2-methylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (45). Compound **45** was synthesized from 2-amino-4-oxo-6-(2-methylbenzyl)-pyrrolo[2,3-*d*]pyrimidine **25** (200 mg, 0.78 mmol) using the general procedure described above to afford **45** 60 mg (28%) as a light yellow solid. Mp: 250 °C. TLC R_f 0.53 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.91 (s, 2H, CH_2), 5.69 (s, 1H, C5-CH), 6.42 (s, 2H, NH_2), 7.13–7.16 (m, 4H, Ar–H), 11.46 (s, 1H, NH).

2-Amino-4-chloro-6-(4-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (46). Compound **46** was synthesized from 2-amino-4-oxo-6-(4-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **27** (137 mg, 0.5 mmol) using the general procedure described above to afford **46** 51 mg (35%) as a light yellow solid. Mp: >250 °C. TLC R_f 0.52 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.93 (s, 2H, CH_2), 5.91 (s, 1H, C5-CH), 6.40 (s, 2H, NH_2), 7.28–7.38 (dd, 4H, Ar–H), 11.45 (s, 1H, NH).

2-Amino-4-chloro-6-(2,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (47). Compound **47** was synthesized from 2-amino-4-oxo-6-(2,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **29** (300 mg, 0.97 mmol) using the general procedure described above to afford **47** 95 mg (30%) as a light yellow solid. Mp: >255 °C. TLC R_f 0.52 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 4.17 (s, 2H, CH_2), 5.82 (s, 1H, C5-CH), 6.48 (s, 2H, NH_2), 7.33–7.66 (m, 3H, Ar–H), 11.53 (s, 1H, NH).

2-Amino-4-chloro-6-(1-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (48). Compound **48** was synthesized from 2-amino-4-oxo-6-(1-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine **31** (400 mg, 1.37 mmol) using the general procedure described above to afford **48** 125 mg (30%) as an off white solid. Mp: >250 °C. TLC R_f 0.53 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 4.11 (s, 2H, CH_2), 5.95 (s, 1H, C5-CH), 6.40 (s, 2H, NH_2), 7.44–7.89 (m, 7H, Ar–H), 11.50 (s, 1H, NH).

4-Amino-4-chloro-6-(4-phenylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (49). Compound **49** was synthesized from 2-amino-4-oxo-6-(4-phenylbenzyl)-pyrrolo[2,3-*d*]pyrimidine **33** (300 mg, 0.95 mmol) using the general procedure described above to afford **49** 98 mg (31%) as a light yellow solid. Mp: >260 °C. TLC R_f 0.53 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.77 (s, 2H, CH_2), 5.75 (s, 1H, C5-CH), 6.22 (s, 2H, NH_2), 7.11–7.26 (m, 5H, Ar–H), 7.39–7.44 (dd, 4H, Ar–H), 11.30 (s, 1H, NH).

General procedure for the synthesis of compounds 5, 7, 9, 11

2-Pivaloylamino-4-chloro-6-substituted-pyrrolo[2,3-*d*]pyrimidines **39–42** (about 0.2 mmol), 3-bromo-aniline **50** (1.5 equivalence), *i*-PrOH 10 mL, and 2–3 drops of concd HCl were placed in a 50-mL flask. The mixture was heated to reflux 45 min. After evaporation of the solvent, the solid was dissolved in 1,4-dioxane 10 mL, and 2 mL of 15% KOH aqueous solution was added. The reaction mixture was heated to reflux for 10 h. After remove of the solvent to form a syrup, water (20 mL) was added and extracted with chloroform (3×50 mL) Combined the organic phase and dried with anhydrous Na_2SO_4. Concentration of the chloroform afforded a solid that was dissolved in methanol, and then 250 mg silica gel was added and removed the solvent in vacuo to afford a dry plug. This plug was placed on the top of a 15×150 mm column and eluted with 2% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford pure 2-amino-4-(3-bromoanilino)-6-subsituted-pyrrolo[2,3-*d*]pyrimidine **5**, **7**, **9**, and **11**.

2-Amino-4-(3-bromoanilino)-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (5). Compound **5** was synthesized from 2-pivaloylamino-4-chloro-6-(2,5-dimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **40** (80 mg, 0.20 mmol) using the general procedure described above to afford 5 50 mg (55%) as an off white solid. Mp: 190–192 °C. TLC R_f 0.43 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.67 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.83 (s, 2H, CH_2), 5.73 (s, 2H, NH_2), 6.06 (s, 1H, C5-CH), 6.75–7.18 (m, 5H, Ar–H), 8.00–8.10 (m, 2H, Ar–H), 8.89 (s, 1H, NH), 10.86 (s, 1H, NH). HRMS (EI): calcd for $C_{21}H_{20}N_5BrO_2$ 453.0800, found 453.0778.

2-Amino-4-(3-bromoanilino)-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (7). Compound **7** was synthesized from 2-pivaloylamino-4-chloro-6-(3,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **41** (100 mg, 0.24 mmol) using the general procedure described above to afford **7** 68 mg (61%) as an off white solid. Mp: 215–217.5 °C. TLC R_f 0.46 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 3.93 (s, 2H, CH_2), 5.77 (s, 2H, NH_2), 6.13 (s, 1H, C5-CH), 7.05–7.60 (m, 5H, Ar–H), 7.99–8.10 (m, 2H, Ar–H), 8.92 (s, 1H, NH), 10.94 (s, 1H, NH). HRMS (EI): calcd for $C_{19}H_{14}N_5BrCl_2$ 460.9810, found 460.9805.

2-Amino-4-(3-bromoanilino)-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (9). Compound **9** was synthesized from 2-pivaloylamino-4-chloro-6-(2-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine **39** (160 mg, 0.4 mmol) using the general procedure described above to afford **9** 130 mg (73%) as an off white solid. Mp: 207–210 °C. TLC R_f 0.49 ($CHCl_3$/CH_3OH, 10:1). ^1H NMR (DMSO-d_6): δ 4.09 (s, 2H, CH_2), 5.77 (s, 2H, NH_2), 6.13 (s, 1H, C5-CH), 7.04–7.51 (m, 9H, Ar–H), 7.99–8.09 (m, 2H, Ar–H), 8.89 (s, 1H, NH), 11.00 (s, 1H, NH). Anal. ($C_{23}H_{18}N_5Br$): C, H, N, Br.

2-Amino-4-(3-bromoanilino)-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine (11). Compound **11** was synthesized from 2-pivaloylamino-4-chloro-6-(3,4,5-trimethoxybenzyl)-pyrrolo[2,3-*d*]pyrimidine **42** (100 mg, 0.23 mmol) using the general procedure described above to afford **11** 67 mg (60%) as an off white solid. Mp:

213–215 °C. TLC R_f 0.42 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 3.62 (s, 3H, OCH_3), 3.69 (s, 6H, 2×OCH_3), 3.84 (s, 2H, CH_2), 5.76 (s, 2H, NH_2), 6.16 (s, 1H, C5-CH), 6.63 (s, 2H, Ar–H), 7.02–7.22 (m, 2H, Ar–H), 8.00–8.11 (m, 2H, Ar–H), 8.93 (s, 1H, NH), 10.93 (s, 1H, NH). Anal. ($C_{22}H_{22}N_5O_3Br$): C, H, N, Br.

General procedure for the synthesis of compounds 1, 2, 3, 4, 6, 8, and 10

2-Amino-4-chloro-6-sustituted-pyrrolo[2,3-*d*]pyrimidines **43–49** (about 0.2 mmol), 3-bromo-aniline **50** (1.5 equiv), *i*-PrOH 10 mL, and 2–3 drops of concd HCl were placed in a 50-mL flask. The mixture was heated to reflux 1.5 h. After being cooled, the reaction mixture was neutralized with $NH_3 \cdot H_2O$, and then the solvent was evaporated in vacuo. The residue solid was dissolved in methanol, then 250 mg silica gel was added and removed the solvent in vacuo to afford a dry plug. This plug was placed on the top of a 15×150 mm column and eluted with 2% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford pure 2-amino-4-(3-bromoanilino)-6-subsituted-pyrrolo[2,3-*d*]pyrimidine **1**, **2**, **3**, **4**, **6**, **8**, **10**.

2-Amino-4-(3-bromoanilino)-6-benzyl-pyrrolo[2,3-*d*]pyrimidine (1). Compound **1** was synthesized from 6-phenyl methyl 2-amino-4-chloro-6-benzyl-pyrrolo[2,3-*d*]pyrimidine **44** (80 mg, 0.30 mmol) using the general procedure described above to afford **1** 90 mg (77%) as an off white solid. Mp: 195–197.5 °C. TLC R_f 0.46 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.05 (s, 2H, CH_2), 5.77 (s, 2H, NH_2), 6.13 (s, 1H, C5-CH), 7.05–7.34 (m, 7H, Ar–H), 7.98–8.11 (m, 2H, Ar–H), 8.93 (s, 1H, NH), 10.93 (s, 1H, NH). HRMS (EI): calcd for $C_{19}H_{16}N_5Br$ 393.0589, found 393.0572.

2-Amino-4-(3-bromoanilino)-6-(2-methylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (2). Compound **2** was synthesized from 2-amino-4-chloro-6-(2-methylbenzyl)-pyrrolo[2,3-*d*]pyrimidine **45** (50 mg, 0.18 mmol) using the general procedure described above to afford **2** 56 mg (75%) as an off white solid. Mp: 225–228 °C. TLC R_f 0.50 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 2.27 (s, 3H, CH_3), 3.89 (s, 2H, CH_2), 5.73 (s, 2H, NH_2), 5.98 (s, 1H, C5-CH), 7.04–7.20 (m, 6H, Ar–H), 7.99–8.09 (m, 2H, Ar–H), 8.87 (s, 1H, NH), 10.91 (s, 1H, NH). Anal. ($C_{20}H_{18}N_5Br$): C, H, N, Br.

2-Aimno-4-(3-bromoanilino)-6-(2-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (3). Compound **3** was synthesized from 2-amino-4-chloro-6-(2-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **43** (35 mg, 0.12 mmol) using the general procedure described above to afford **3** 45 mg, 88%) as an off white solid. Mp: 208–211 °C. TLC R_f 0.47 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 3.91 (s, 2H, CH_2), 5.77 (s, 2H, NH_2), 6.10 (s, 1H, C5-CH), 7.05–7.45 (m, 6H, Ar–H), 8.00–8.10 (m, 2H, Ar–H), 8.91 (s, 1H, NH), 10.94 (s, 1H, NH). HRMS (EI): calcd for $C_{19}H_{15}N_5BrCl$ 427.0199, found 427.0218.

2-Amino-4-(3-bromoanilino)-6-(4-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (4). Compound **4** was synthesized from 2-amino-4-chloro-6-(4-chlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **46** (30 mg, 0.1 mmol) using the general procedure described above to afford **4** 36 mg (82%) as an off white solid. Mp: 195–197.5 °C. TLC R_f 0.45 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.03 (s, 2H, CH_2), 5.78 (s, 2H, NH_2), 6.07 (s, 1H, C5-CH), 7.05–7.49 (m, 6H, Ar–H), 8.00–8.10 (m, 2H, Ar–H), 8.92 (s, 1H, NH), 10.97 (s, 1H, NH). Anal. ($C_{19}H_{15}N_5BrCl \cdot 0.2(CH_3)_2CHOH$): C, H, N, Br, Cl.

2-Amino-4-(3-bromoanilino)-6-(2,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine (6). Compound **6** was synthesized from 2-amino-4-chloro-6-(2,4-dichlorobenzyl)-pyrrolo[2,3-*d*]pyrimidine **47** (80 mg, 0.24 mmol) using the general procedure described above to afford **6** 77 mg (70%) as a light yellow solid. Mp: 196–198 °C. TLC R_f 0.44 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.01 (s, 2H, CH_2), 5.81 (s, 2H, NH_2), 6.04 (s, 1H, C5-CH), 7.05–7.64 (m, 5H, Ar–H), 8.00–8.09 (m, 2H, Ar–H), 8.91 (s, 1H, NH), 10.99 (s, 1H, NH). HRMS (EI): calcd for $C_{19}H_{14}N_5BrCl_2$ 460.9810, found 460.9790.

2-Amino-4-(3-bromoanilino)-6-(1-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine (8). Compound **8** was synthesized from 2-amino-4-chloro-6-(1-naphthylmethyl)-pyrrolo[2,3-*d*]pyrimidine **48** (50 mg, 0.16 mmol) using the general procedure described above to afford **8** 50 mg (71%) as an off white solid. Mp: 235–238 °C. TLC R_f 0.51 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.38 (s, 2H, CH_2), 5.73 (s, 2H, NH), 5.96 (s, 1H, C5-CH), 7.02-7.52 (m, 9H, Ar–H), 7.83–8.04 (m, 2H, Ar–H), 8.81 (s, 1H, NH), 11.02 (s, 1H, NH). HRMS (EI): calcd for $C_{23}H_{18}N_5Br$ 443.0746, found 443.0758.

2-Amino-4-(3-bromoanilino)-6-(4-phenylbenzyl)-pyrrolo[2,3-*d*]pyrimidine (10). Compound **10** was synthesized from 2-amino-4-chloro-6-(4-phenylbenzyl)-pyrrolo[2,3-*d*]pyrimidine **49** (50 mg, 0.15 mmol) using the general procedure described above to afford **10** 58 mg (83%) as white solid. Mp: 200–202 °C. TLC R_f 0.50 ($CHCl_3/CH_3OH$, 10:1). ^1H NMR (DMSO-d_6): δ 4.04 (s, 2H, CH_2), 5.85 (s, 2H, NH_2), 6.18 (s, 1H, C5-CH), 7.06–7.71 (m, 11H, Ar–H), 7.95–8.10 (m, 2H, Ar–H), 8.99 (s, 1H, NH), 11.01 (s, 1H, NH). Anal. ($C_{25}H_{20}N_5Br$): C, H, N, Br.

Cells

All cells were maintained at 37° C in a humidified environment containing 5% CO_2 using media from Mediatech (Hemden, NJ, USA). A-431 cells were from the American Type Tissue Collection (Manassas, VA, USA).

Chemicals

All growth factors (bFGF, VEGF, EGF, PDGF-BB) were purchased from Peprotech (Rocky Hill, NJ, USA). PD153035, SU5416, AG1295 and VEGF kinase inhibitor (4-[4′-chloro-2′-fluoro)phenylamino]-6,7-dimethoxyquinazoline) were purchased from Calbiochem (San Diego, CA, USA). The CYQUANT cells proliferation assay was from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma Chemical unless otherwise noted.

Antibodies

The PY-HRP antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Antibodies against EGFR, PDGFRβ, FGFR-1, Flk-1, and Flt-1 were purchased from Upstate Biotech (Framingham, MA, USA).

Phosphotyrosine (PY) ELISA

Cells used were tumor cell lines naturally expressing high levels of EGFR (A431), Flk-1 (U251), Flt-1 (A498), and PDGFR β (SF-539) and FGFR-1 (NIH OVCAR-8). Expression levels at the RNA level were derived from the NCI Developmental Therapeutics Program (NCI-DTP) web site public molecular target information (http://www.dtp.nci.nih.gov/mtargets/mt_index.html). 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 are then pre-treated for 60 min with 10, 3.33, 1.11, 0.37 and 0.12 μM compound followed by 100 ng/mL EGF, VEGF, PDGF-BB, or bFGF for 10 min. The reaction is stopped and cells permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05% triton X-100, protease inhibitor cocktail and tyrosine phosphatase inhibitor cocktail. The TBS solution is then removed and cells fixed to the plate by 30 min at 60 °C and further incubation in 70% ethanol for an additional 30 min. Cells are further exposed to block (TBS with 1% BSA) for 1 h, washed, and then a horseradish peroxidase (HRP)-conjugated phosphotyrosine antibody added overnight. The antibody is removed, cells are washed again in TBS, exposed to an enhanced luminol ELISA substrate (Pierce Chemical, Rockford, IL, USA) and light emission measured using an UV Products (Upland, CA, USA) BioChemi digital darkroom. The known RTK-specific kinase inhibitor PD153035 was used as a positive control compound for EGFR kinase inhibition, SU5416 for Flk1 kinase inhibition. AG1295 for PDGFRβ kinase inhibition, and VEGF kinase inhibitor (4 - [4′ - chloro - 2′ - fluoro)phenylamino] - 6,7 - dimethoxyquinazoline) was used as a positive control for both Flt1 and Flk1 kinase inhibition. Data were graphed as a percent of cells receiving growth factor alone and IC_{50} values estimated from 2–3 separate experiments ($n = 8$–24) using hand-drawn Probit plots. In every case, the activity of a positive control inhibitor did not deviate more than 10% from the IC_{50} values listed in the text.

CYQUANT cell proliferation assay

As a measure of cell proliferation, the CYQUANT cell counting/proliferation assay was used as previously described.[68] Briefly, cells are first treated with compounds for 12 h and allowed to grow for an additional 36 h. The cells are then lysed and the CYQUANT dye, which intercalates into the DNA of cells, is added and after 5 min the fluorescence of each well measured using an UV Products BioChemi digital darkroom. A Positive control used for cytotoxicity in each experiment was cisplatin, with an apparent average IC_{50} value of 8.2 ± 0.65 μM. Data are graphed as a percent of cells receiving growth factor alone and IC_{50} values estimated from 2–3 separate experiments ($n = 6$–15) using Probit plots.

Chorioallantoic membrane (CAM) assay of angiogenesis

The CAM assay is a standard assay for testing antiangiogenic agents.[69] The CAM assay used in these studies was modified from a procedure by Sheu[74] and Brooks[75] and as published previously.[76] Briefly, fertile leghorn chicken eggs (CBT Farms, Chestertown, MD, USA) are allowed to grow until 10 days of incubation. The proangiogenic factors human VEGF-165 and bFGF (100 ng each) are then added to saturation to a 6-mm microbial testing disc (BBL, Cockeysville, MD, USA) and placed onto the CAM by breaking a small hole in the superior surface of the egg. Antiangiogenic compounds are added 8 h after the VEGF/bFGF at saturation to the same microbial testing disk and embryos allowed to incubate for an additional 40 h. After 48 h, the CAMs are perfused with 2% paraformaldehyde/3% glutaraldehyde containing 0.025% Triton X-100 for 20 s, excised around the area of treatment, fixed again in 2% paraformaldehyde/3% glutaraldehyde for 30 min, placed onto Petri dishes, and a digitized image taken using a dissecting microscope (Wild M400; Bannockburn, IL, USA) at 7.5× and SPOT Enhanced digital imaging system (Diagnostic Instruments, Sterling Heights, MI, USA). A grid is then added to the digital CAM images and the average number of vessels within 5–7 grids counted as a measure of vascularity. AGM-1470 (a kind gift of the NIH Developmental Therapeutics Program) and SU5416 are used as a positive control for antiangiogenic activity. Data are graphed as a percent of CAMs receiving bFGF/VEGF and IC_{50} values estimated from 2–3 separate experiments ($n = 5$–11) using Probit plots.

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Appendix

Elemental Analyses

Compd	Formula	Calcd (%)					Found (%)				
		C	H	N	Cl	Br	C	H	N	Cl	Br
24	$C_{13}H_{12}N_4O$	64.99	5.03	23.32			64.97	5.10	23.08		
25	$C_{14}H_{14}N_4O$	66.13	5.55	22.03			66.16	5.61	21.89		
26	$C_{13}H_{11}N_4OCl{\cdot}0.8H_2O$	54.01	4.39	19.38	12.26		53.93	4.50	19.16	12.44	
27	$C_{13}H_{11}N_4OCl$	56.84	4.04	20.39	12.91		56.93	4.12	20.15	12.82	
28	$C_{15}H_{16}N_4O_3$	59.99	5.37	18.66			59.69	5.36	18.37		
29	$C_{13}H_{10}N_4OCl_2{\cdot}0.6H_2O$	48.80	3.53	17.51	22.16		48.58	3.53	17.47	22.01	
30	$C_{13}H_{10}N_4OCl_2{\cdot}0.1CH_3OH$	50.37	3.36	17.94	22.70		50.64	3.49	17.62	22.45	
31	$C_{17}H_{14}N_4O{\cdot}0.4H_2O$	68.63	5.01	18.83			68.65	4.83	18.71		
32	$C_{17}H_{14}N_4O{\cdot}0.8H_2O$	67.00	5.16	18.39			66.96	5.00	18.03		
33	$C_{19}H_{16}N_4O{\cdot}0.1H_2O$	71.73	5.13	17.61			71.60	5.14	17.40		
34	$C_{16}H_{18}N_4O_4$	58.17	5.49	16.96			58.15	5.58	16.86		
35	$C_{22}H_{22}N_4O_2{\cdot}0.3H_2O$	69.38	6.25	14.71			69.16	6.00	14.82		
39	$C_{22}H_{21}N_4OCl$	67.26	5.39	14.26	9.02		66.98	5.42	14.12	9.27	
43	$C_{13}H_{10}N_4Cl_2{\cdot}0.2H_2O$	52.62	3.53	18.88	23.89		52.88	3.46	18.54	24.02	
2	$C_{20}H_{18}N_5Br$	58.83	4.44	17.15		19.57	58.57	4.52	17.15		19.86
4	$C_{19}H_{15}N_5BrCl{\cdot}0.2(CH_3)_2CHOH$	53.41	3.80	15.89	8.04	18.13	53.70	3.73	16.15	8.00	18.03
9	$C_{23}H_{18}N_5Br$	62.17	4.08	15.76		17.98	62.40	4.10	15.46		18.29
10	$C_{25}H_{20}N_5Br$	63.84	4.29	14.89		16.99	63.72	4.36	14.63		17.22
11	$C_{22}H_{22}N_5O_3Br$	54.56	4.58	14.46		16.50	54.28	4.59	14.30		16.70

High-Resolution Mass Spectra (HRMS) (EI)

Compd	Formula	Calcd mass	Found mass
1	$C_{19}H_{16}N_5Br$	393.0589	393.0572
3	$C_{19}H_{15}N_5BrCl$	427.0199	427.0218
5	$C_{21}H_{20}N_5BrO_2$	453.0800	453.0778
6	$C_{19}H_{14}N_5BrCl_2$	460.9810	460.9790
7	$C_{19}H_{14}N_5BrCl_2$	460.9810	460.9805
8	$C_{23}H_{18}N_5Br$	443.0746	443.0758

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