Published in final edited form as:

Bioorg Med Chem. 2013 April 1; 21(7): 1857–1864. doi:10.1016/j.bmc.2013.01.040.

Synthesis and biological activity of 5-chloro-*N*⁴-substituted phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamines as vascular endothelial growth factor receptor-2 inhibitors and antiangiogenic agents¹

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Abstract

Inhibition of receptor tyrosine kinase (RTK) signaling pathways is an important area for the development of novel anticancer agents. Numerous multikinase inhibitors (MKIs) have been recently approved for the treatment of cancer. Vascular endothelial growth factor receptor-2 (VEGFR-2) is the principal mediator of tumor angiogenesis. In an effort to develop ATP-competitive VEGFR-2 selective inhibitors the 5-chloro-*N*⁴-substituted phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine scaffold was designed. The synthesis of the target compounds involved *N*-(4,5-dichloro-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide as a common intermediate. A nucleophilic displacement of the 4-chloro group of the common intermediate by appropriately substituted anilines afforded the target compounds. Biological evaluation indicated that compound **5** is a potent and selective VEGFR-2 inhibitor comparable to sunitinib and semaxinib.

Keywords

Receptor tyrosine kinase inhibitors; Pyrimido[4, 5-*b*]indol synthesis; Cytotoxicity; VEGFR-2 inhibitors

1. Introduction

Protein tyrosine kinases are a key means of signal transduction in eukaryotic cells and control processes such as cell proliferation, differentiation, migration, survival, and cell cycle progression. Dysregulation of these tightly regulated processes through overexpression of kinases is implicated in numerous disease states including cancer.^{2,3}

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Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature and this process is crucial for a solid tumor to grow beyond 1–2 mm. As a tumor grows in size, it becomes increasingly hypoxic, leading to induction of growth factors including vascular endothelial growth factor (VEGF), and other important proangiogenic regulators like epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), transforming growth factors (TGF α and β), and tumor necrosis factor- α (TNF- α) among others.^{2, 4} The growth factors function by binding to receptor tyrosine kinases (RTKs), a super family of transmembrane proteins. Subsequent to binding of the growth factor, RTKs dimerize and undergo autophosphorylation, initiating angiogenesis.⁵ Anti-angiogenic therapy targets non-tumor cells (endothelial cells) which may be less prone to mutations and hence to resistance compared to tumor cells.⁶

One of the approaches to circumvent angiogenesis is the inhibition of the key RTKs involved in angiogenesis. Several reports on multikinase inhibitors (MKIs) have appeared in the recent literature, some of which have afforded clinically approved agents. Imatinib (Figure 1) was the first RTK inhibitor approved in 2001 for chronic myelogenous leukemia (CML).^{7,8} Imatinib inhibits Abelson Tyrosine Kinase (Abl), c-kit protein (CD117), PDGFR- α and PDGFR- β .

Sorafenib, approved in 2005 for the treatment of hepatocellular carcinoma and renal cell carcinoma, inhibits Raf kinase, VEGFR-2, VEGFR-3, PDGFR- β , and c-kit.⁹ Sunitinib, approved in 2006 for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors, inhibits VEGFR, PDGFR, c-kit, and FMS-like tyrosine kinase 3 (FLT3).¹⁰

Two drugs – dasatinib¹¹ and nilotinib¹² were approved in 2006 and 2007 respectively, specifically for imatinib-resistant or unresponsive tumors in CML. Dasatinib inhibits Abl, c-Kit, PDGFR, and Src. Nilotinib inhibits Abl, c-Kit, PDGFR- β , Src, and Ephrin. Lapatinib, approved in 2007 for advanced metastatic breast cancer in conjunction with chemotherapy, inhibits EGFR and erythroblastic leukemia viral oncogene homolog-2 (ErbB-2).¹³

Although the approval of numerous MKIs for cancer therapy attests to the importance of this approach in cancer chemotherapy, gaining selectivity for a limited subset of kinases for MKIs is a widely recognized challenge facing medicinal chemists in the kinase area.¹⁴ The risk of a MKI approach is that such compounds are by their very nature more likely to hit a diverse spectrum of kinases. A recent report studied the selectivity of approved kinase inhibitors and candidates across 317 different kinases.¹⁵ The most selective of the currently approved MKIs was lapatinib, and the least selective was sunitinib; the latter bound > 15% of kinases tested with $K_d < 100$ nM. Bamborough et al.¹⁶ screened 577 diverse compounds versus 203 protein kinases and found that two-thirds of the compounds bound to more than 10 kinases, hence clearly demonstrating the extent of the selectivity challenge in the kinase area.

Where a large number of closely related kinase isozymes exist, some of which may be crucial for normal cellular function, it is critical to avoid the off-target activity of MKIs as it might translate into undesirable biology. This has been exemplified by the approved MKIs sorafenib and sunitinib which have been reported to be cardiotoxic.^{17,18} In addition, imatinib has been reported to have mechanism-based cardiotoxic effects, and nilotinib carries a black box warning for possible heart complications.¹⁹ Hence, specific inhibitors of kinases critical in tumor survival like VEGFR-2 or other RTKs, with minimal off-target activity, are also of considerable interest.

In 1996, Traxler et al.²⁰ reported a series of *N*⁴-substituted phenyl-9*H*-pyrimido[4,5-*b*]indoles as inhibitors of EGFR. From this series, compound **1** (Figure 2) emerged as a single digit nanomolar inhibitors of EGFR (IC₅₀ = 6 nM). Showalter et al.²¹ reported several elaborations of similar anilino pyrimidine inhibitors of which compound **2** emerged as another potent inhibitor of EGFR (IC₅₀ = 147 nM). Compounds **1** and **2** were not tested in VEGFR-2, which has been implicated as the principal mediator of angiogenesis^{22,23–25} and VEGFR-2 stimulation alone is enough to initiate tumor growth and metastases.²⁵ It was of interest to structurally design a scaffold to afford either dual EGFR/VEGFR-2 inhibition or perhaps a selective VEGFR-2 inhibition.

On the basis of **1** and **2**, we designed general scaffold A – 5-chloro-*N*⁴-substituted phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine (Figure 2). A systematic conformational search (5° increments) performed using Sybyl X 1.3²⁶ indicated a lower number of conformations (24) about the C⁴–N bond with the pyrimidine ring for 5-chloro-*N*⁴-phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine **3** (Figure 5) compared to its corresponding 5-deschloro analog, **2** (41). Hence, the addition of a 5-Cl substitution was expected to provide conformational restriction through steric hindrance to rotation and this may allow for specificity against some kinases over others and perhaps allow potent inhibition of a different spectrum of RTKs.

Molecular modeling was carried out to gain some insight to the binding mode of **3** in the VEGFR-2 active site. The best scored pose on docking compound **3** using Lead IT 2.1.0²⁷ is depicted in Figures 3 and 4. The binding site of ATP competitive inhibitors in RTKs consists of a Hinge region, and a hydrophobic binding site (Hydrophobic Region I) among others (Figure 3).^{28–31} The pyrimido[4,5-*b*]indole ring of **3** occupies the adenine binding portion of the ATP binding site. The 1-N and the 9-NH are involved in hydrogen bonds with Cys917 in the Hinge region. Hydrophobic interactions of the indole ring with Val914, Phe916, Cys917, Leu1033, Cys1043, Leu838 and Val846 can stabilize the docked pose. In addition, the aniline phenyl extends towards Hydrophobic region I and is involved in interactions with Val846, Ala864, Lys866, Val897, Val914 and Leu1033. The 2-amino group could potentially form a hydrogen bond with Glu915 in the hinge region.

A series of six compounds **3–8** (Figure 5) was designed to study scaffold A with respect to inhibition of receptor tyrosine kinases. The selection of the substituents on the 4-position are rationalized below. The 4'-isopropyl group in **4** was included to obtain information about bulk tolerance in this position in the RTK active site. Compound **15** (bearing a 4-chloroaniline) reported by Bold et al.³² demonstrated potent VEGFR-2 and VEGFR-1 inhibition in **15** (Figure 5). Thus compound **5** with the 4'-Cl aniline was proposed with the purpose of obtaining potent inhibition of VEGFR-2 and/or VEGFR-1. The 3'-OMe aniline substitution in **8** is similar to compounds **16** and **17** (Figure 5) which are potent inhibitors of EGFR.³³ In order to obtain further SAR information at this position, the 3'-OMe group (**8**) was replaced with the 3'-F (**7**) which would afford information regarding electron withdrawing groups at the 3'-position. Compound **6** with the 2'-F, 4'-Cl aniline substituted ring is similar to that in **18**³⁴ and was included with the purpose of obtaining potent inhibition of VEGFR-2 and VEGFR-1 as reported for **18**. The unsubstituted phenyl ring (**3**) served as a comparison with the substituted compounds.

The synthesis of **3–8** was designed from the corresponding 2-amino pivaloyl (2,2-dimethylpropan-1-one) protected precursors **9–14**. It was decided to biologically evaluate intermediates **9–14** to study the effect of the 2-amino pivaloyl groups on binding to RTKs. It was anticipated that the pivaloyl moieties of **9–14** could interact at hydrophobic region II³⁵ and perhaps afford increased activity against some RTK.

2. Chemistry

Compounds **3–14** were synthesized as described in Scheme 1. *N*-(4,5-dichloro-9*H*-pyrimido[4,5-*b*]indol-2-yl)pivalamide **19** was synthesized by a method developed by Gangjee et al.³⁵ Compound **19** was condensed with appropriately substituted anilines in isopropanol at reflux, in the presence of three drops of conc. HCl, to afford regiospecifically **9–14**. Deprotection of the 2-pivaloyl group in **11** by reaction with 15% KOH in 1,4-dioxane at reflux afforded **5**. Compounds **9**, **10** and **12** were more soluble in a 1:1 mixture of methanol to methylene chloride than in 1,4-dioxane. However, when 1 N NaOH and the 1:1 methanol/methylene chloride solvent system were used for the deprotection reaction of **9**, **10** and **12** to obtain **3**, **4** and **6** respectively, the result was an increased reaction time (1–3 days) and lower yields for **3** (52%) and **4** (49%) (This method afforded **6** in 74% yield). Reaction time was significantly decreased when 1 N NaOH in isopropanol was used as the reaction system; **13** and **14** were converted to **7** (70%) and **8** (92%) respectively in 2–14 h. Isopropanol was more efficient, probably because of the enhanced solubility of the starting material (**13**, **14**) as well as the nucleophile (hydroxide ions).

3. Biological evaluation and discussion

Compounds **3–14** were evaluated as RTK inhibitors using human tumor cells known to express high levels of VEGFR-2, EGFR and PDGFR- β using a phosphotyrosine ELISA cyto blot (Table 1).³⁶ Whole cell assays were used for RTK inhibitory activity since these assays afford more meaningful results for translation to in vivo studies. The effect of compounds on cell proliferation was measured using A431 cancer cells, known to overexpress EGFR. EGFR is known to play a role in the overall survival of A431 cells.³⁶

Since the IC₅₀ values of compounds vary under different assay conditions (e.g., ATP concentrations), standard compounds (Figure 6) were used as controls in each of the evaluations. The standard compounds used were semaxanib (SU5416), **20** for VEGFR-2;³⁷ 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline (PD153035), **21** for EGFR;³⁸ 3-(4-dimethylamino-benzylidenyl)-2-indolinone (DMBI), **22** for PDGFR- β .³⁹ and cisplatin, **23** for A431 cytotoxicity. Approved agents sunitinib (a multitargeted RTK inhibitor including VEGFR-2, PDGFRs) and erlotinib⁴⁰ (a specific EGFR inhibitor approved for non small cell lung cancer) were also used as standard agents.

In the VEGFR-2 kinase inhibition assay, of the target compounds **3–8**, the 4-chlorophenyl substituted compound **5** was the most active and equipotent to the standard **20**. Compound **5** was also 1.4- and 9.4-fold more potent than sunitinib and erlotinib respectively in the VEGFR-2 inhibition assay. The next most potent compounds were the 4-isopropylphenyl substituted **4**, and the 2-fluoro-4-chlorophenyl substituted **6** which were 2.5-fold less active and 3-fold less active respectively, than **20**. VEGFR-2 inhibition decreased on moving the phenyl substitution from the 4- to the 3-position, as exemplified by the 3-fluorophenyl substituted **7** and the 3-methoxy substituted **8**, which were 6- and 7-fold less potent than **20** respectively.

For the 2-amino pivaloyl substituted compounds **9–14**, the 4-chlorophenyl substituted compound **11** was the most active and was 2-fold less potent than the standard **20** in the VEGFR-2 inhibition assay. The next most potent compounds included the 3-fluorophenyl substituted **13**, 3-methoxyphenyl substituted **14**, and the 4-isopropylphenyl substituted **10**, which were about 2.5-, 3.5- and 4-fold less active than **20**, respectively. The increased activity of the 2-pivaloyl analogs **13–14** in the VEGFR-2 assay over the corresponding despivaloyl analogs **7** and **8** respectively indicates a possible interaction with hydrophobic site II of the pivaloyl moiety in VEGFR-2.

A trend in activity was observed for **3–5**, however in that these compounds were about 1.5-fold more active than their 2-amino pivaloyl derivatives. However, a similar trend was not observed for **7** and **8** which were about 2.8- and 1.7-fold less active than the corresponding 2-amino pivaloyl compounds **13** and **14**. Thus subtle differences in binding orientation of **3–5** compared with **7–8** perhaps accounts for the differences in their respective pivaloyl derivatives.

The most potent compound against EGFR was the 2-amino pivaloyl, 3-fluorophenyl substituted **13** which was 150-fold less potent than the standard **21**. However compound **13** was 5.5-fold more active than sunitinib, and 25-fold less active than erlotinib in the EGFR inhibition assay. The presence of a 5-chloro group is detrimental to cell-based EGFR inhibition relative to the 5-deschloro lead compound **2** (Figure 2) which is a reported nanomolar EGFR inhibitor in an isolated enzyme assay. The most potent PDGFR- β inhibitor in this study was the 3-methoxyphenyl substituted **8**, which is about 13.5-fold less active than the standard **22**. Compound **8** was 1.6-fold more potent than sunitinib and 4-fold less active than erlotinib in the PDGFR- β inhibition assay.

In the A431 cytotoxicity assay, the most potent compounds were the 4-isopropylphenyl substituted **4**, and the 4-chlorophenyl substituted **5** which were 4-fold less potent than the standard **23**. Since substitution at any other position produced a further decrease in activity, the 4-position is optimum for activity, and tolerates both electron withdrawing and electron donating as well as bulky groups. Of the 2-amino pivaloyl derivatives **9–14**, only the 2-amino pivaloyl, 4-chlorophenyl substituted **11** produced potent inhibition, which was 5-fold less than the standard **23**.

In summary, compounds **3–14** were synthesized to develop a novel scaffold with either selective VEGFR-2 and/or VEGFR-1 inhibition or EGFR/VEGFR-2 dual inhibition. The cellular inhibition assays demonstrated that the compounds were indeed selective for VEGFR-2, relative to EGFR and PDGFR- β . The 4-chlorophenyl substituted **5** was the most potent inhibitor in the VEGFR-2 inhibition assay and was equipotent with the standard compound **20**, and was 1.4- and 9.4-fold more potent than sunitinib and erlotinib respectively. The results of this study indicate that the 5-chloro-*N*⁴-substituted phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine scaffold is highly selective for VEGFR-2 inhibition with minimal off-target kinase inhibition in the limited number of RTKs these compounds were evaluated against and serves as a new lead analog that is equipotent to sunitinib and almost 10x more potent than erlotinib in the VEGFR-2 inhibition whole cell assay.

4. Experimental section

Analytical samples were dried in vacuo (0.2 mm Hg) in a CHEM-DRY drying apparatus over P₂O₅ at 80 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (¹H NMR) were recorded on a Bruker WH-300 (300 MHz) or Bruker WH-400 (400 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million): s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within $\pm 0.4\%$ of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be prevented in spite of 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. or Fisher Scientific and were used as received.

General procedure for the synthesis of 9–14

To a 50 mL round bottom flask were added *N*-(4,5-dichloro-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethyl propanamide **19**, various substituted anilines, isopropanol (50 mL) followed by 3 drops of concd HCl. The mixture was heated at reflux for 1.5 to 6 h. The solvent was removed by evaporation in vacuo. The residue was dissolved in methanol and silica gel (2 g) was added followed by evaporation in vacuo to afford a dry silica gel plug which was loaded on top of a wet (hexane) silica gel column sequentially eluting with 0%, 1% and 2% methanol in chloroform. Fractions containing desired product (TLC) were pooled and evaporated to dryness to afford the product.

***N*-(4-anilino-5-chloro-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide (9)**—Reaction of **19** (150 mg, 0.44 mmol) and aniline (310 mg, 3.33 mmol) using the general procedure described above, gave **9**: yield 86%, TLC R_f 0.6 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 249–250 °C; ¹H NMR (DMSO-*d*₆) δ 1.27 (s, 9 H, C(CH₃)₃), 7.03–7.08 (m, 1 H, Ar), 7.33–7.38 (m, 4 H, Ar), 7.46–7.49 (m, 1 H, Ar), 8.13–8.16 (d, 2 H, Ar), 9.36 (s, 1 H, 2-NH, exch), 9.67 (s, 1 H, 4-NH, exch), 12.25 (s, 1 H, 9-NH, exch). Anal. (C₂₁H₂₀ClN₅O) C, H, N, Cl.

***N*-(5-chloro-4-[(4-isopropylphenyl)amino]-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide (10)**—Reaction of **19** (250 mg, 0.74 mmol) and 4-isopropyl aniline (750 mg, 5.56 mmol) using the general procedure described above, gave **10**: yield 75%, TLC R_f 0.62 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 265 °C; ¹H NMR (DMSO-*d*₆) δ 1.20 (s, 3 H, CH₃), 1.23 (s, 3 H, CH₃), 1.27 (s, 9 H, C(CH₃)₃), 7.20–7.23 (m, 1 H, Ar), 7.35–7.40 (m, 2 H, Ar), 7.46–7.49 (m, 1 H, Ar), 8.03–8.06 (d, 2 H, Ar), 9.31 (s, 1 H, 2-NH, exch), 9.63 (s, 1 H, 4-NH, exch), 12.24 (s, 1 H, 9-NH, exch). HRMS (ESI) [M+H]⁺ calcd. for C₂₄H₂₆ClN₅O m/z = 436.1904, found m/z = 436.1868.

***N*-(5-chloro-4-[(4-chlorophenyl)amino]-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide (11)**—Reaction of **19** (150 mg, 0.44 mmol) and 4-chloro aniline (188 mg, 1.46 mmol) using the general procedure described above, gave **11**: yield 48%, TLC R_f 0.5 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 281.8–282 °C; ¹H NMR (DMSO-*d*₆) δ 1.26 (s, 9 H, C(CH₃)₃), 7.31–7.40 (m, 4 H, Ar), 7.45–7.48 (m, 1 H, Ar), 8.19–8.22 (d, 2 H, Ar), 9.37 (s, 1 H, 2-NH, exch), 9.70 (s, 1 H, 4-NH, exch), 12.25 (s, 1 H, 9-NH, exch). Anal. (C₂₁H₁₉Cl₂N₅O) C, H, N, Cl.

***N*-(5-chloro-4-[(4-chloro, 2-fluorophenyl)amino]-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide (12)**—Reaction of **19** (150 mg, 0.44 mmol) and 2-fluoro-4-chloroaniline (485 mg, 3.33 mmol) using the general procedure described above, gave **12**: yield 74%, TLC R_f 0.6 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 284–285 °C; ¹H NMR (DMSO-*d*₆) δ 1.28 (s, 9 H, C(CH₃)₃), 7.23–7.26 (m, 1 H, Ar), 7.33–7.40 (m, 2 H, Ar), 7.49–7.57 (m, 2 H, Ar), 9.47–9.53 (m, 1 H, Ar), 9.50 (s, 1 H, 2-NH, exch), 9.82 (s, 1 H, 4-NH, exch), 12.34 (s, 1 H, 9-NH, exch). Anal. (C₂₁H₁₈Cl₂FN₅O) 0.025 CHCl₃) C, H, N, Cl, F.

***N*-(5-chloro-4-[(3-fluorophenyl)amino]-9*H*-pyrimido[4,5-*b*]indol-2-yl)-2,2-dimethylpropanamide (13)**—Reaction of **19** (100 mg, 0.29 mmol) and 3-fluoroaniline (242 mg, 2.17 mmol) using the general procedure described above, gave **13**: yield 95%, TLC R_f 0.6 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 273–274 °C; ¹H NMR (DMSO-*d*₆) δ 1.28 (s, 9 H, C(CH₃)₃), 6.82–6.89 (m, 1 H, Ar), 7.33–7.39 (m, 3 H, Ar),

7.48–7.54 (s, 2 H, Ar), 8.61–8.66 (d, 1 H, Ar), 9.46 (s, 1 H, 2-NH, exch), 9.79 (s, 1 H, 4-NH, exch), 12.27 (s, 1 H, 9-NH, exch). Anal. (C₂₁H₁₉ClFN₅O) C, H, N, Cl, F.

N-[5-chloro-4-[(3-methoxyphenyl)amino]-9H-pyrimido[4,5-b]indol-2-yl]-2,2-dimethylpropanamide (14)—Reaction of **19** (100 mg, 0.29 mmol) and 3-methoxyaniline (268 mg, 2.17 mmol) using the general procedure described above, gave **14**: yield 91%, TLC *R_f* 0.6 (CHCl₃/MeOH, 10:1 with 2 drops concentrated NH₄OH); mp 196–197 °C; ¹H NMR (DMSO-*d*₆) δ 1.26 (s, 9 H, C(CH₃)₃), 3.86 (s, 3 H, CH₃), 6.61–6.63 (m, 1 H, Ar), 7.19–7.28 (m, 2 H, Ar), 7.31–7.44 (m, 2 H, Ar), 7.48–7.50 (m, 1 H, Ar), 8.18 (s, 1 H, Ar), 9.37 (s, 1 H, 2-NH, exch), 9.79 (s, 1 H, 4-NH, exch), 12.26 (s, 1 H, 9-NH, exch). Anal. (C₂₂H₂₂ClN₅O₂ · 0.09 CHCl₃) C, H, N, Cl.

5-chloro-N⁴-(4-chlorophenyl)-9H-pyrimido[4,5-b]indole-2,4-diamine (5)—In a 50 mL flask **11** (70 mg, 0.16 mmol) was dissolved in 1,4-dioxane 20 mL, and 2 mL of 15% KOH aqueous solution was added. The reaction mixture was heated to reflux for 14 h. The solvent was evaporated to obtain a residue. Water was added and the mixture was extracted with chloroform. Sodium sulfate was used to dry the extract. Silica gel was added and solvent evaporated to make a plug. The plug was loaded on top of a wet (CHCl₃) silica gel column (25 cm × 3 cm) and eluted with 60:1 (CHCl₃: CH₃OH). Fractions containing desired product were pooled and evaporated to afford **5** in 95% yield. TLC *R_f* 0.35 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 306.1–307 °C; ¹H NMR (DMSO-*d*₆) δ 6.54 (bs, 2 H, NH₂, exch), 7.19–7.21 (m, 2 H, Ar), 7.29–7.37 (m, 4 H, Ar), 7.95–7.98 (m, 2 H, Ar), 9.12 (s, 1 H, 4-NH, exch), 11.75 (s, 1 H, 9-NH, exch). HRMS (ESI) [M+H]⁺ calcd. for C₁₆H₁₁Cl₂N₅ *m/z* = 344.0470, found *m/z* = 344.0449.

5-chloro-N⁴-(4-isopropylphenyl)-9H-pyrimido[4,5-b]indole-2,4-diamine (4)—In a 50 mL flask **10** (20 mg, 0.044 mmol) was dissolved in CH₂Cl₂/CH₃OH, 1:1 (10 mL), and 2 mL of 1 N NaOH solution was added. The reaction mixture was heated to reflux for 44 h. The solvent was evaporated to obtain a residue. Water was added and the mixture was filtered and dried. The solid was dissolved in MeOH, silica gel was added and solvent evaporated to make a plug. The plug was loaded on top of a wet (CHCl₃) silica gel column and eluted with 20:1 (CHCl₃: MeOH). Fractions containing desired product were pooled and evaporated to afford the **4** in 52% yield. TLC *R_f* 0.40 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 235–236 °C; ¹H NMR (DMSO-*d*₆) δ 1.19–1.21 (m, 6 H, CH₃ × 2), 2.85–2.90 (m, 1 H, CH), 6.42 (bs, 2 H, NH₂, exch), 7.17–7.20 (m, 4 H, Ar), 7.27–7.30 (m, 2 H, Ar), 7.75–7.77 (m, 2 H, Ar), 9.00 (s, 1 H, 4-NH, exch), 11.68 (s, 1 H, 9-NH, exch). Anal. (C₁₉H₁₈ClN₅) C, H, N, Cl.

5-chloro-N⁴-phenyl-9H-pyrimido[4,5-b]indole-2,4-diamine (3)—Compound **3** (synthesized from **9** (130 mg, 0.33 mmol) as described for **4**; reaction time = 15 h): yield 49%; TLC *R_f* 0.40 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 271–272 °C; ¹H NMR (DMSO-*d*₆) δ 6.48 (bs, 2 H, NH₂, exch), 7.01–7.04 (m, 1 H, Ar), 7.18–7.19 (m, 2 H, Ar), 7.29–7.34 (m, 3 H, Ar), 7.89–7.91 (d, 2 H, Ar), 9.08 (s, 1 H, 4-NH, exch), 11.71 (s, 1 H, 9-NH, exch). Anal. (C₁₆H₁₂ClN₅ · 0.04 CHCl₃) C, H, N, Cl.

5-chloro-N⁴-(4-chloro-2-fluorophenyl)-9H-pyrimido[4,5-b]indole-2,4-diamine (6)—Compound **6** (synthesized from **12** (100 mg, 0.22 mmol) as described for **4**; reaction time = 48 h): yield 74%; TLC *R_f* 0.40 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 296 °C; ¹H NMR (DMSO-*d*₆) δ 6.61 (s, 2 H, NH₂, exch), 7.21–7.33 (m, 4 H, Ar), 7.52–7.55 (m, 1 H, Ar), 8.91–8.96 (m, 1 H, Ar), 9.14 (s, 1 H, 4-NH, exch), 11.82 (s, 1 H, 9-NH, exch). HRMS (ESI) [M+H]⁺ calcd. for C₁₆H₁₁Cl₂FN₅ *m/z* = 362.0376, found *m/z* = 362.0381.

5-chloro-*N*⁴-(3-fluorophenyl)-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine (7)—In a 50 mL flask **13** (84 mg, 0.20 mmol) was dissolved in isopropanol (50 mL), and 2 mL of 1 N NaOH solution was added. The reaction mixture was heated to reflux for 14 h. The solvent was evaporated to obtain a residue. Water was added and the mixture was filtered and dried. The solid was dissolved in CH₃OH, silica gel was added and solvent evaporated to make a plug. The plug was loaded on top of a wet (CHCl₃) silica gel column and eluted with 30:1 (CHCl₃: MeOH). Fractions containing desired product were pooled and evaporated to afford the **7** in 70% yield. TLC *R*_f 0.47 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 257–258 °C; ¹H NMR (DMSO-*d*₆) δ 6.64 (bs, 2 H, NH₂, exch), 6.81–6.86 (m, 1 H, Ar), 7.20–7.22 (m, 2 H, Ar), 7.30–7.37 (m, 2 H, Ar), 7.43–7.46 (m, 1 H, Ar), 8.13–8.16 (m, 1 H, Ar), 9.21 (s, 1 H, 4-NH, exch), 11.79 (s, 1 H, 9-NH, exch). Anal. (C₁₆H₁₁ClFN₅·0.5 H₂O) C, H, N, Cl, F.

5-chloro-*N*⁴-(3-methoxyphenyl)-9*H*-pyrimido[4,5-*b*]indole-2,4-diamine (8)—Compound **8** (synthesized from **14** (85 mg, 0.20 mmol) as described for **7**; reaction time = 2 h): yield 92%; TLC *R*_f 0.42 (CHCl₃/MeOH, 10:1 with 3 drops concentrated NH₄OH); mp 242–243 °C; ¹H NMR (DMSO-*d*₆) δ 3.78 (s, 3 H, CH₃), 6.48 (bs, 2 H, NH₂, exch), 6.59–6.62 (m, 1 H, Ar), 7.18–7.23 (m, 3 H, Ar), 7.27–7.31 (m, 2 H, Ar), 7.66 (m, 1 H, Ar), 9.06 (s, 1 H, 4-NH, exch), 11.72 (s, 1 H, 9-NH, exch). Anal. (C₁₇H₁₄ClN₅O) C, H, N, Cl.

Cells

All cells were maintained at 37 °C in a humidified environment containing 5% CO₂ using media from Mediatech (Hemden, NJ). A-431 cells were from the American Type Tissue Collection (Manassas, VA).

Chemicals

All growth factors (bFGF, VEGF, EGF, and PDGF-β) were purchased from Peprotech (Rocky Hill, NJ). PD153035, SU5416, AG1295, and CB676475 (4-[(4'-chloro-2'-fluoro)phenylamino]-6,7-dimethoxyquinazoline) were purchased from Calbiochem (San Diego, CA). The CYQUANT cell proliferation assay was from Molecular Probes (Eugene, OR). All other chemicals were from Sigma Chemical unless otherwise noted.

Antibodies

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

Phosphotyrosine ELISA

Cells used were tumor cell lines naturally expressing high levels of EGFR (A431), Flk-1 (U251), Flt-1 (A498), 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 were 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 10, 3.33, 1.11, 0.37, and 0.12 μM compounds followed by 100 ng/ml EGF, VEGF, or PDGF-BB for 10 min. The reaction was stopped and cells permeabilized by quickly removing the media from the cells and adding ice-cold Tris-buffered saline (TBS) containing 0.05% Triton X-100, protease inhibitor cocktail, and tyrosine phosphatase inhibitor cocktail. The TBS solution was then removed and cells fixed to the plate for 30 min at 60 °C 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 added overnight. The antibody was removed, cells were washed again in TBS, exposed to an enhanced luminal ELISA substrate (Pierce Chemical, Rockford, IL), and light emission measured using a UV product (Upland, CA) 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 PDGFR- β kinase inhibition; and CB676475 (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 IC₅₀ values were determined from two to three separate experiments ($n = 8-24$) using non-linear regression dose-response analysis with Prism 5.0 software (GraphPad, San Diego, CA). In each case, the activity of a positive control inhibitor did not deviate more than 10% from the IC₅₀ 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.³⁶ Briefly, cells are first treated with compounds for 12 h and then 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 a BioTek plate reader (Biotek, Winooski, VT). A positive control used for cytotoxicity in each experiment was cisplatin, **23**. Data are graphed as a percent of cells receiving growth factor alone and IC₅₀ values determined from two to three separate experiments ($n = 6-15$) using non-linear regression dose-response analysis with Prism 5.0 software (GraphPad, San Diego, CA).

Statistics

All analysis was done using Prism 5.0. (GraphPad Software, San Diego, CA).

Molecular Modeling and Computational Studies

The X-ray crystal structure of VEGFR2 at 1.71 Å resolution was obtained from the protein database (PDB ID 1YWN)⁴¹. This crystal structure contains VEGFR2 in complex with a novel 4-amino-furo[2,3-*d*]pyrimidine. Docking studies were performed using Lead IT.²⁷ The protein was prepared in Lead IT using default settings. Docking with Lead IT was carried out in by defining the active site as a sphere of ~6.5 Å from the X-ray crystal structure ligand. The protonation states utilized for the proteins and the ligands were calculated using the default settings. Water molecules in the active site were permitted to rotate freely. Ligands for docking were prepared using MOE 2010.10⁴² and energy minimized using the MMFF94X forcefield to a constant of 0.05 kcal/mol. Triangle matching was used as the placement method and the docked poses were scored using default settings. The docked poses were exported and visualized in MOE. The docking protocol was validated by re-docking the X-ray crystal structure ligand. The best docked pose in the re-docking study had an RMSD of 0.7686 Å. Docking studies were performed for 3 in Lead IT using a similar procedure. Poses from the docking experiment performed in Lead IT were exported and visualized in MOE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in part, by the National Institutes of Health and National Cancer Institute Grant CA98850 (A.G.) and the Duquesne University Adrian Van Kaam Chair in Scholarly Excellence (AG).

Abbreviations

ATP	adenosine 5' triphosphate
VEGF	vascular endothelial growth factor
EGF	epidermal growth factor
PDGF	platelet derived growth factor
RTK	receptor tyrosine kinase
VEGFR-2	vascular endothelial growth factor receptor-2
VEGFR-1	vascular endothelial growth factor receptor-1
PDGFR-β	platelet derived growth factor receptor- β
FGF	fibroblast growth factor
IGF-1	insulin-like growth factor-1
TGFα and β	transforming growth factors
TNF-α	tumor necrosis factor- α
MKIs	multikinase inhibitors
CML	chronic myelogenous leukemia
Abl	Abelson Tyrosine Kinase
CD117	c-kit protein
FLT3	FMS-like tyrosine kinase 3

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Figure 1.
Various RTK inhibitors

Figure 2.
Design of scaffold (I)

Figure 3.
Stereoview of docked pose of compound **3** in VEGFR-2 (PDB code: 1YWN) showing the key interactions.

Figure 4.
Ligand interaction plot of the docked pose of compound **3** in VEGFR-2 (PDB code: 1YWN) showing the key interactions.

Figure 5. 5-Chloro- N^4 -substituted phenyl-9*H*-pyrimido[4,5-*b*]indole-2,4-diamines as potential multiple RTK inhibitors with lead compounds

Figure 6.

Scheme 1.

Reagents and conditions: (a) ArNH₂, Isopropanol, 3 drops of conc. HCl, reflux, 1.5–6 h (48%–95%); (b) 15% KOH, 1,4-dioxane, reflux, 14 h (95%); (c) 1 N NaOH, methanol/CH₂Cl₂, reflux, 1–3 d (49%–74%); (d) 1 N NaOH, Isopropanol, reflux, 2–14 h (70%–92%).

Table 1IC₅₀ values (μM) of kinase inhibition and A431 cytotoxicity for compounds **3–14**

Compound	VEGFR-2 kinase inhibition	EGFR Kinase Inhibition	PDGFR-β kinase inhibition	A431 cytotoxicity
3	123.8 ± 29.0	>200	>200	193.1 ± 29.7
4	30.3 ± 5.0	42 ± 10.5	>200	45.1 ± 10.1
5	13.2 ± 1.7	63.6 ± 8.1	196.2 ± 22.4	44.1
6	38.9 ± 6.0	38.9 ± 6.0	>200	128.3 ± 20.2
7	86.1 ± 7.8	>200	172.2 ± 30.1	184.1 ± 22.1
8	70.1 ± 10.1	82.9 ± 19.2	50.5 ± 7.2	167.0 ± 38.6
9	192.0 ± 16.2	114.7 ± 30.2	70.6 ± 10.7	185.2 ± 20.1
10	47.8 ± 10.1	39.2 ± 7.8	>200	>250
11	21.3 ± 4.0	58.7 ± 7.2	>200	55.0
12	89.1 ± 10.9	>200	170.8 ± 19.6	>250
13	31.2 ± 4.5	30.8 ± 7.0	>200	>250
14	41.0 ± 7.2	130.5 ± 32.8	196.2 ± 30.0	>250
Semaxanib(20)	12.0 ± 2.7			
PD153035 (21)		0.2 ± 0.004		
DMBI (22)			3.7 ± 0.06	
Sunitinib	18.9 ± 2.7	172.1 ± 19.4	83.1 ± 10.1	
Erlotinib	124.7 ± 18.2	1.2 ± 0.2	12.2 ± 1.9	
Cisplatin (23)				10.6 ± 2.9