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N^4-Aryl-6-substitutedphenylmethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines as receptor tyrosine kinase inhibitors

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ABSTRACT

Six novel N^4-substitutedphenyl-6-substitutedphenylmethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines were synthesized as multiple receptor tyrosine kinase (RTK) inhibitors and antitumor agents. An improvement in the inhibitory potency against epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) assays and in the A431 cellular proliferation assay was observed for compounds **8–13** over the previously reported **5–7**. Three compounds (**8**, **9** and **13**) demonstrated potent, multiple RTK inhibition and were more potent or equipotent compared to the lead compounds **5** and **7** and the standard compounds. Compounds **10** and **12** showed potent inhibition of VEGFR-2 over EGFR, platelet-derived growth factor receptor-β (PDGFR-β) and VEGFR-1. The results indicate that the RTK inhibitory profile could be modulated with slight variations to the N^4-aryl-6-substitutedphenylmethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamino scaffold.

1. Introduction

Dysfunctional receptor tyrosine kinases (RTKs) have been associated with several cancers where they play a pivotal role in tumor angiogenesis.[1,2] Angiogenesis requires the transduction of signals from the extracellular domain of endothelial cells to the nucleus which is mediated by RTKs.[3] Solid tumors require angiogenesis to grow beyond 1–2 mm in size and metastasis requires the presence of blood vessels to allow transport of tumor cells to sites distal to the primary tumor.[3,4] Inhibition of tumor angiogenesis prevents the growth and metastasis of several types of solid tumors. Thus, inhibition of angiogenesis via RTK inhibition provides an attractive target for the treatment of cancer.[1,5] Among the RTKs implicated in tumor progression and angiogenesis are members of the VEGFR family namely VEGFR-1 and VEGFR-2, members of the EGFR family and members of the PDGFR family, namely PDGFR-α and PDGFR-β.[5,6]

Small molecule RTK inhibitors targeting the ATP binding site of tyrosine kinases are currently in clinical use while others are in clinical trials as antitumor agents.[5,7,8] Initial strategies for RTK inhibition focused on single RTK inhibitors such as erlotinib, **1** and gefitinib, **2** that were approved for non small cell lung cancer (Fig. 1).[7,8] However, tumors have redundant signaling pathways for angiogenesis and often develop resistance to agents that target one specific pathway.[9,10] A multitargeted approach that inhibits multiple signaling pathways has shown to be more effective than the inhibition of a single target.[10,11] Sorafenib, **3** an inhibitor of VEGFR, PDGFR and Raf-1 kinase and sunitinib, **4** an inhibitor of VEGFR-1, VEGFR-2, fms-like tyrosine kinase-3 (Flt-3), PDGFR, stem cell factor receptor (c-Kit) and colony stimulating factor (CSF-1) have been approved for renal cell carcinoma, and sunitinib most recently for pancreatic cancer.[12,13]

Gangjee et. al.[14] previously reported compounds **5–7** (Fig. 2) as multiple RTK inhibitors in a series of N^4-(3-bromophenyl)-6-substitutedphenylmethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines. It was demonstrated that variation of the phenyl substituents in the 6-benzyl moiety determined both the potency and specificity of inhibitory activity against various RTKs. To further develop the structure–activity relationship, it was of interest to determine if variation in the anilino moiety could similarly influence potency and specificity for RTK inhibition. Thus, compounds **8–13** (Fig. 2) were synthesized as analogs of **5–7** with two different 4-anilino moieties in combination with the 6-benzyl sidechains of **5–7**. We elected the 4-chloro anilino and 4-chloro-2-fluoroanilino substitutions in compounds **8–13** on the basis of the potent multiple RTK inhibition seen for these anilines in 6–6 fused systems such as quinazolines, pthalazines and pyrido[2,3-*d*]pyrimidines.[15–19] Vatalinib bearing the 4-chloro anilino substitution, has shown potent VEGFR-1 and VEGFR-2 inhibition,[15,16] while quinazolines bearing the 4-chloro-2-fluoroanilino substitution have shown potent, dual VEGFR-2 and EGFR inhibition.[17,18] The 2-NH_2 moiety in **8–13** was maintained to provide additional

Abbreviations: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; CSF-1, colony stimulating factor; PDGFR, platelet-derived growth factor receptors.

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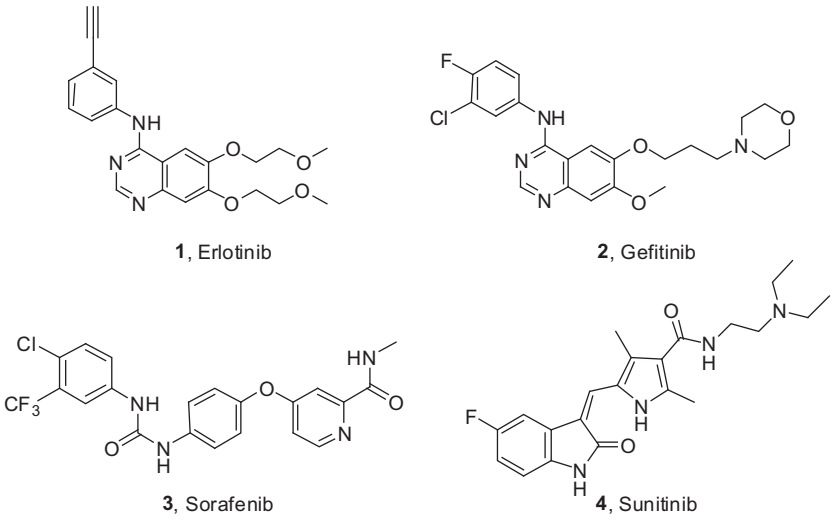
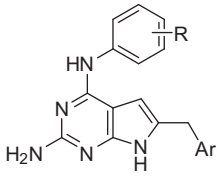
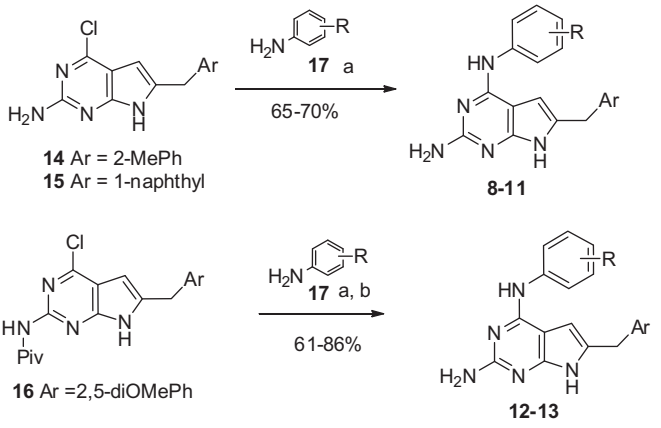


Figure 1. Reported RTK inhibitors.



	R	Ar
5	3-Br	2-MePh
6	3-Br	2,3-C_4H_4Ph
7	3-Br	2,5-diOMePh
8	2-F, 4-Cl	2-MePh
9	4-Cl	2-MePh
10	2-F, 4-Cl	2,3-C_4H_4Ph
11	4-Cl	2,3-C_4H_4Ph
12	2-F, 4-Cl	2,5-diOMePh
13	4-Cl	2,5-diOMePh

Figure 2. Target compounds **8**–**13** as analogs of **5**–**7**.



Scheme 1. Reagents and conditions: (a) **17**, *i*PrOH, 2–3 drops concd HCl, 4 h, reflux; (b) KOH, 1,4-dioxane, reflux.

hydrogen bonding in the Hinge region of RTKs compared to other known RTK inhibitors that lack this 2-NH_2 moiety. The flexible 6-benzyl substitutions were incorporated to allow for multiple conformations of this side chain and to perhaps afford interactions with multiple RTKs.

2. Chemistry

The synthesis of compounds **8–13** is shown in Scheme 1. The 4-chloro-6-substituted pyrrolo[2,3-*d*]pyrimidines, **14–15**[14] were synthesized in five steps from the corresponding phenylacetic acids. Treatment with the appropriate aniline, **17** in isopropanol and a few drops of concd HCl at reflux afforded compounds **8–11**. Reaction of **16**[14] (synthesized in six steps from 2,5-dimethoxyphenylacetic acid) with the appropriate aniline **17** in isopropanol and a few drops of concd HCl, followed by depivaloylation with base, at reflux afforded compounds **12–13**.

3. Results and discussions

The RTK inhibitory activities of compounds **8–13** were evaluated in human tumor cells known to express high levels of EGFR, VEGFR-2, VEGFR-1 and PDFGR-β using a phosphotyrosine ELISA assay.[19] The effect of compounds **8–13** 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.[19] Cellular evaluations of RTK inhibitory activities afford more meaningful results for translation to in vivo studies than direct enzymatic assays. Since the IC_{50} values of compounds vary under different assay conditions, standard compounds **18–22** (Fig. 3) were used as controls in each of the evaluations. The standard compounds used were semaxanib, **18** for VEGFR-2[20]; **19**, (4-chloro-2-fluorophenyl)-6,7-dimethoxy quinazolin-4-yl-amine (CB676475) for VEGFR-1[17]; **20**, 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline (PD153035) for EGFR[21]; and **21**, 3-(4-dimethylamino-benzylidenyl)-2-indolinone (DMBI) for PDGFR-β.[22] Cisplatin, **22** was used as the standard for the A431 cytoxicity assay. For a better demonstration of the kinase inhibitory potential of the target compounds described in this study, two clinically approved RTK inhibitors, erlotinib, **1** and sunitinib, **4** were incorporated for comparison.[7,13] The inhibitory potencies (IC_{50} values) of **8–13** are also compared with the previously synthesized compounds **5–7** and standard compounds **1**, **4**, **18–22** in Table 1.

In the 2-methylphenylsubstituted compounds, **9** with the 4-chloro anilino substitution showed potent EGFR inhibition, being equipotent to the standard compound, PD153035, fivefold more potent than erlotinib and 38-fold better than compound **5**. EGFR inhibition decreased for the 2-fluoro-4-chloro anilino derivative **8** compared to **5**. In the 6-(1-naphthyl)substituted compounds, variation of the 3-bromo anilino moiety to the 2-fluoro-4-chloro anilino in **10** and the 4-chloro anilino substitution in **11** did not improve EGFR inhibition. For the 2,5-dimethoxyphenylsubstituted pyrrolo[2,3-*d*]pyrimidines, EGFR inhibition further decreased on variation to the 2-fluoro-4-chloro anilino substitution in **12** and the 4-chloro anilino substitution in **13**.

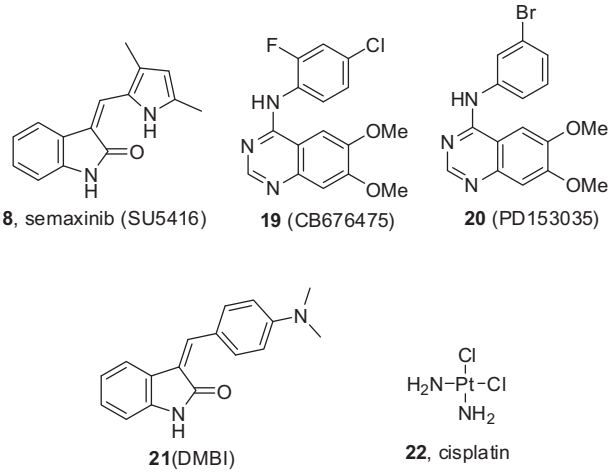


Figure 3. Reported RTK Inhibitors used as standard compounds for comparison in the cellular evaluation of **8**–**13**.

In the VEGFR-2 assay, the variation of the 3-bromo anilino substitution to the 2-fluoro-4-chloro anilino in compound **8** and the 4-chloro anilino substitution in **9** showed a decrease in activity compared to **5** among the 6-(2-methylphenyl)substituted pyrrolo[2,3-*d*]pyrimidines. In the 6-(1-naphthyl)substituted compounds, the VEGFR-2 inhibitory activity improved in the 2-fluoro-4-chloro anilino substituted compound **10** compared to **6** and was 25-fold more potent than semaxanib, and 39-fold more potent than sunitinib, **4**. Compound **11** with the 4-chloro anilino substitution showed moderate VEGFR-2 inhibition and was approximately threefold and twofold less potent compared to semaxanib and sunitinib, respectively. In the 2,5-dimethoxyphenylsubstituted compounds, the VEGFR-2 inhibition improved with variation of the 2-fluoro-4-chloro anilino moiety in compound **12**, but decreased on variation to the 4-chloro anilino moiety in **13**. Compound **12** was remarkably 120-fold and 189-fold more potent against VEGFR-2 compared to semaxanib and sunitinib, respectively.

In the VEGFR-1 assay, inhibitory potency improved with variation to the 2-fluoro-4-chloro anilino moiety in the 2-methylphenyl-substituted compound **8** compared to **5** and was almost equipotent to the standard CB676475. The 4-chloro anilino substituted compound **9** was also almost equipotent to **5** and showed a fourfold lower inhibition of VEGFR-1 compared to CB676475. In the 6-(1-naphthyl)substituted compounds, variation to the 2-fluoro-4-chloro anilino in **10** and the 4-chloro anilino substitution in **11** decreased VEGFR-1 inhibition compared to **6** and the standard CB676475. Among the 2,5-dimethoxyphenylsubstituted compounds, VEGFR-1 inhibition significantly decreased for the 2-fluoro-4-chloro anilino substituted compound **12** compared to **7** and CB676475, and improved for the 4-chloro anilino compound **13**. Compound **13** showed a twofold better and almost equipotent VEGFR-1 inhibition compared to **7** and CB676475, respectively.

In the PDGFR-β assay, variation of the 3-bromo anilino moiety to the 2-fluoro-4-chloro anilino and the 4-chloro anilino substitution did not provide PDGFR-β inhibition in the 6-(2-methylphenyl) substituted or 6-(1-naphthyl)substituted compounds **8–11**. Among the 2,5-dimethoxyphenylsubstituted compounds, the PDGFR-β inhibitory activity decreased with variation in the anilino moiety in **12** and **13** compared to **7**.

A431 cytotoxicity significantly decreased for the 2-fluoro-4-chloro anilino substituted compounds **8** and **10**, while moderate cytoxicity in the A431 proliferation assay was seen for the 4-chloro anilino substituted compounds **9** and **11**. Compound **9** was twofold more potent than cisplatin and approximately fivefold less potent than **5**. Compound **11** showed a fourfold better inhibition compared to **6**, and was almost equipotent to cisplatin. In the 2,5-dimethoxyphenylsubstituted compounds, an improvement in A431 cytotoxicity was observed on variation in the anilino moiety. Both compounds **12** and **13** were better or equivalent to the standard cisplatin, and fourfold and eightfold better than **7**, respectively.

Multiple kinase inhibitory profiles were observed for compounds **8**, **9** and **13**. Compound **8** was a dual VEGFR-1 and VEGFR-2 inhibitor. Compound **9** demonstrated potent EGFR, moderate VEGFR-1 and VEGFR-2 inhibition and potent A431 cytotoxicity. Compound **13** demonstrated potent VEGFR-1 inhibition, A431 cytotoxicity and moderate VEGFR-2 inhibition. Submicromolar, specific inhibition of VEGFR-2 over EGFR, VEGFR-1 and PDGFR-β was observed for compounds **10** and **12**. Interestingly, compounds **11–13** showed potent A431 cytoxicities although they did not show significant EGFR inhibition. The A431 cell lines depend on EGFR for survival; perhaps these compounds do not directly inhibit EGFR but influence the downstream signaling of EGFR and crosstalk with other kinases which may be necessary for the functioning of EGFR.[14] There have been several literature reports in which the EGFR inhibitory activity does not translate into the A431 cytotoxicity.[23,24] It will be of interest to determine whether another kinase (other than EGFR) also plays a role in A431 cell survival. Since the inhibitory activities are determined in cells, it is not possible to make a definite conclusion on the structure–activity relationship for compounds **8–13**.

Table 1
IC_{50} values (μM) of kinase inhibition and A431 cytotoxicity for compounds **8**–**13**

Cpd	EGFR	VEGFR-2	VEGFR-1	PDGFR-β	A431 cytotoxicity
5[a]	9.19 ± 1.8	0.25 ± 0.04	>50	>50	1.21 ± 0.42
8	>200	29.8 ± 5.0	16 ± 1.9	>300	>200
9	0.24 ± 0.031	45.4 ± 6.2	45.9 ± 4.3	>300	5.6 ± 0.08
6[a]	>50	5.08 ± 0.83	19.2 ± 4.3	>50	>50
10	>200	0.48 ± 0.06	>200	193.2 ± 20.1	46.7 ± 6.2
11	112.1 ± 18.2	32.1 ± 5.2	>200	>300	8.5 ± 0.93
7[a]	12.62 ± 3.3	0.62 ± 0.21	31.1 ± 5.8	8.92 ± 1.6	>50
12	112.7 ± 20.2	0.1 ± 0.021	185.6 ± 27.5	145.9 ± 23.8	13.4 ± 0.21
13	>200	56.7 ± 5.1	15.2 ± 0.26	44.3 ± 5.2	5.8 ± 0.62
Semaxanib		12.0 ± 2.7			
CB676475			14.1 ± 2.8		
PD153035	0.23 ± 0.04				
DMBI				3.75 ± 0.06	
Cisplatin					10.6 ± 2.9
Erlotinib	1.2 ± 0.2	124.7 ± 18.2		83.1 ± 10.1	
Sunitinib	172.1 ± 19.4	18.9 ± 2.7		12.2 ± 1.9	

[a] IC_{50} values from Ref. 14.

4. Conclusion

We synthesized six novel RTK inhibitors to determine the effect of substitution in the 4-anilino ring along with variations in the 6-substituent of the pyrrolo[2,3-*d*]pyrimidine scaffold. Compound **12** emerged as the most viable candidate for future evaluation with its remarkable 120-fold and 189-fold increased potency against VEGFR-2 compared to semaxinib and sunitinib, respectively. Several analogs (**8, 9** and **13**) demonstrated multiple RTK inhibition while others (**10** and **12**) were specific for VEGFR-2. The cytotoxicity against A431 cells in culture was somewhat better for some compounds (**9, 11** and **13**) compared with cisplatin, although **9**, **11** and **13** did not show improved EGFR inhibition. Compounds **9, 11** and **13** are interesting candidates for further evaluation against additional kinases.

Our results indicate that the potency and selectivity of cellular inhibition of different RTKs does indeed vary with different anilino substitutions and that an optimal combination of the substitutions in the 4-anilino ring and the 6-benzyl substituent is essential for RTK inhibition of the N^4-aryl-6-substituted phenylmethyl-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines.

5. Experimental section

5.1. Synthesis

Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P_2O_5 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 (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz) or a Bruker 400 MHz/52 MM (400 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer or in a LKB-9000 instrument in the electron ionization (EI) or electron spray (ESI) mode. Chemical names follow IUPAC nomenclature. 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 0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented in spite of 24–48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. Microwave-assisted synthesis was performed utilizing an Emrys Liberator microwave synthesizer (Biotage) utilizing capped reaction vials. All microwave reactions were performed with temperature control. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received.

5.1.1. N^4-(4-Chloro-2-fluorophenyl)-6-(2-methylbenzyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (8)

To a 100 mL round-bottom flask was added **14** (100 mg, 0.44 mmol), 2-fluoro-4-chloroaniline (1.5 equiv), *i*PrOH (20 mL) and 6 drops of concd HCl. The mixture was refluxed for 12 h. After being cooled, the reaction mixture was dried in vacuo. The residue was neutralized with NH_4OH (1 mL) and extracted with $CHCl_3$ (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to afford a yellow solid. The crude product was purified by flash chromatography on silica gel (gradient, $CHCl_3$ to 2% MeOH/$CHCl_3$) to afford 80 mg (70%) of **8** as a white solid; TLC R_f 0.54 ($CHCl_3$/CH_3OH, 10:1); mp 210 °C; ^{1}H NMR (DMSO-d_6) δ 2.29 (s, 3H, CH_3), 3.89 (s, 2H, CH_2), 5.60 (s, 2H, NH_2), 5.94 (s, 1H, C5–H), 7.16–7.92 (m, 7H, Ar–H), 8.62 (s, 1H, NH), 10.89 (s, 1H, NH). Anal. ($C_{20}H_{17}ClFN_5$·0.08 $CHCl_3$) C, H, N, F, Cl.

5.1.2. N^4-(4-Chlorophenyl)-6-(2-methylbenzyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (9)

Compound **9** was synthesized as described for **8** with 4-chloroaniline and was obtained as an off white solid (65%); TLC R_f 0.52 ($CHCl_3$/CH_3OH, 10:1); mp 212 °C; ^{1}H NMR (DMSO-d_6) δ 2.27 (s, 3H, CH_3), 3.89 (s, 2H, CH_2), 5.99 (s, 1H, C5–H), 5.70 (s, 2H, NH_2), 7.17–7.23 (m, 4H, Ar–H), 7.26(d, 2H, Ar–H), 7.96 (d, 2H, Ar–H), 8.87 (s, 1H, NH), 10.88 (s, 1H, NH). Anal. ($C_{20}H_{18}ClN_5$·0.23 H_2O) C, H, N, Cl.

5.1.3. N^4-(4-Chloro-2-fluorophenyl)-6-(1-naphthylmethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (10)

Compound **10** was synthesized as described for **8** with **15** and was obtained as an off white solid (70%); TLC R_f 0.58 ($CHCl_3$/CH_3OH, 10:1); mp 212 °C; ^{1}H NMR (DMSO-d_6) δ 4.37 (s, 2H, CH_2), 5.91(s, 1H, C5–H), 5.57 (s, 2H, NH_2), 6.55–8.07 (m, 10H, Ar–H), 9.3 (s, 1H, NH), 10.96 (s, 1H, NH). Anal. ($C_{23}H_{18}ClFN_5$) C, H, N, Cl, F.

5.1.4. N^4-(4-Chlorophenyl)-6-(1-naphthylmethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (11)

Compound **11** was synthesized as described for **10** with 4-chloroaniline and was obtained as a white solid (68%); TLC R_f 0.55 ($CHCl_3$/CH_3OH, 10:1); mp 209 °C; ^{1}H NMR (DMSO-d_6) δ 4.38 (s, 2H, CH_2), 5.95 (s, 1H, C5–H), 5.69 (s, 2H, NH_2), 7.45–8.04 (m, 9H, Ar–H), 7.23 (m, 2H, Ar–H), 8.77 (s, 1H, NH), 10.99 (s, 1H, NH). Anal. ($C_{23}H_{18}ClN_5$·0.2 H_2O) C, H, N, Cl.

5.1.5. N^4-(2-Fluoro-4-chlorophenyl)-6-(2,5-dimethoxybenzyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (12)

To a 100 mL round-bottom flask was added **16** (200 mg, 0.49 mmol), 2-fluoro-4-chloroaniline (1.5 equiv), *i*PrOH (20 mL) and 6 drops of concd HCl. The mixture was refluxed for 12 h. After being cooled, the reaction mixture was dried in vacuo. The residue was neutralized with NH_4OH (1 mL) and extracted with $CHCl_3$ (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to afford a yellow solid. To this was added 1,4-dioxane (10 mL) and 15% KOH (2 mL) and the mixture was refluxed for 10 h. After removal of the solvent, water (20 mL) was added, extracted with $CHCl_3$ (30 mL), dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (gradient, $CHCl_3$ to 2% MeOH/$CHCl_3$) to afford 150 mg (86%) of **12** as a light yellow solid; mp 186–187 °C; TLC R_f 0.51 ($CHCl_3$/CH_3OH, 10:1); ^{1}H NMR (DMSO-d_6) δ 3.34 (s, 2H, CH_2), 3.65 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 5.95 (s, 2H, NH_2), 6.81 (s, 1H, CH), 6.94–7.38 (m, 6H, Ar–H), 8.89 (s, 1H, NH), 11.06 (s, 1H, NH). Anal. ($C_{21}H_{19}ClFN_5O_2$·0.9 H_2O) C, H, N, F, Cl.

5.1.6. N^4-(4-Chlorophenyl)-6-(2,5-dimethoxybenzyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamine (13)

Compound **13** was synthesized as described for **12** with 4-chloroaniline and was obtained as a white solid (61%); TLC R_f 0.48 ($CHCl_3$/CH_3OH, 10:1); mp 188 °C; ^{1}H NMR (DMSO-d_6) δ 3.62 (s, 2H, CH_2), 3.73 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 5.87 (s, 2H, NH_2), 6.35 (s, 1H, CH), 6.74–7.15 (m, 3H, Ar–H), 7.22 (d, 2H, Ar–H), 7.44 (d, 2H, Ar–H), 11.03 (s, 1H, NH), 11.41 (s, 1H, NH). Anal. ($C_{21}H_{20}ClN_5O_2$·0.18 H_2O) C, H, N, Cl.

5.2. Biological evaluation

All cells were maintained at 37 °C in a humidified environment containing 5% CO_2 using media from Mediatech (Hemden, NJ, USA). The A-431 cells were from the American Type Tissue Collection (Manassas, VA, USA). All growth factors (bFGF, VEGF, EGF, PDGF-BB) were purchased from Peprotech (Rocky Hill, NJ, USA). The PY-HRP antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Antibodies against EGFR, PDGFR-β, FGFR-1, Flk-1, and Flt-1 were purchased from Upstate Biotech (Framingham, MA, USA). The CYQUANT cell proliferation assay was from Molecular Probes (Eugene, OR, USA).The standard compounds used for comparison in the assays were purchased from Calbiochem (San Diego, CA, USA).

5.3. Inhibition of cellular tyrosine phosphorylation

Inhibition of EGF, VEGF and PDGF-BB-stimulated total cellular tyrosine phosphorylation in tumor cells naturally expressing high levels of EGFR (A431), VEGFR-2 (U251), VEGFR-1 (A498) and PDGFR-β (SF-539), respectively, were measured using the ELISA assay as previously reported.[19] 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 pre-treated for 60 min with 333, 100, 33.3, 10, 3.33, 1.00, 0.33 and 0.10 μM compound followed by 100 ng/mL EGF, VEGF, PDGF-BB, or bFGF 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 by 30 min at 60 °C and further incubated 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 antibody was added overnight. The antibody was removed, cells were 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. Standard compounds were used as controls in each of the evaluations. The standard compounds used were semaxanib, **18** for VEGFR-2; (4-chloro-2-fluorophenyl)-6, 7-dimethoxy quinazolin-4-yl-amine, **19** for VEGFR-1; 4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline, **20** for EGFR; 3-(4-dimethylamino-benzylidenyl)-2-indolinone, **21** for PDGFR-β. Erlotinib, **1** and sunitinib, **4** were also evaluated against VEGFR-2, EGFR and PDGFR-β in this assay. Data were graphed as a percent of cells receiving growth factor alone and IC_{50} values estimated from 2 to 3 separate experiments (n = 8–24) using non-linear regression Sigmoidal Dose–Response analysis with GraphPad Prism (San Diego, CA). 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.

5.4. Antiproliferative assay

The assay was performed as described previously.[19] Briefly, cells were first treated with compounds for 12 h and then allowed to grow for an additional 36 h. The cells were then lysed and the CYQUANT dye, which intercalates into the DNA of cells, was added and after 5 min the fluorescence of each well measured using an UV Products BioChemi digital darkroom. Cisplatin, **22** was used as the standard for cytotoxicity in each experiment. Data were graphed as a percent of cells receiving growth factor alone and IC_{50} values estimated from 2 to 3 separate experiments (n = 6–15) using non-linear regression Sigmoidal Dose-Response analysis with GraphPad Prism (San Diego, CA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.058.

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