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The contribution of a 2-amino group on receptor tyrosine kinase inhibition and antiangiogenic activity in 4-anilinosubstituted pyrrolo[2,3-*d*]pyrimidines

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ABSTRACT

Comparison between a series of pyrrolo[2,3-*d*]pyrimidines with and without the 2-amino group is presented in order to determine the validity of our hypothesis that inclusion of this group improves potency against receptor tyrosine kinases (RTK). The 2-amino analogs were better against epidermal growth factor receptor (EGFR) and platelet derived growth factor-β (PDGFR-β) in whole cell inhibition assays and in the A431 cytotoxicity assay compared to the 2-desamino analogs. However, the 2-desamino analogs were more potent inhibitors against vascular endothelial growth factor-2 (VEGFR-2) than the corresponding 2-amino compounds. In addition, none of the 2-desamino compounds exhibited better anti-angiogenic activity in the chorioallantoic membrane (CAM) assay as compared to the standard and were only micromolar inhibitors. This study validates our original hypothesis that the inclusion of a 2-amino group in pyrrolo[2,3-*d*]pyrimidines improves multiple RTK inhibition and antiangiogenic activity.

Receptor tyrosine kinases (RTKs) are a subfamily of protein tyrosine kinases, which play key roles in tumor growth, survival and dissemination.[1] A variety of growth factors particularly vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and their receptors are overexpressed in several tumors. These growth factors and their receptors are directly or indirectly involved in the growth and metastasis of tumors.[2]

Angiogenesis is the formation of new blood vessels from existing vasculature and is essential for both physiological and pathological processes. It is a complex cascade that is tightly regulated by proangiogenic and antiangiogenic factors.[3] Members of the VEGF family are the predominant stimulators of angiogenesis and mediation of VEGF expression is one of the main mechanisms by which tissue vasculature is controlled under normal physiologic conditions.[4–6] In majority of cancers, in addition to this PDGF subfamilies appear to play essential roles in all stages of tumor angiogenesis and are able to form autocrine loops, which mediate cancer cell growth and survival, and drive hematologic malignancies.[7] Angiogenesis is a pivotal step in the transition of some solid tumors from a dormant state to a malignant state; it also provides a metastatic pathway for solid tumors.[8] In addition, angiogenesis contributes to the development of hematologic malignancies, particularly multiple myeloma, leukemia, and lymphoma. Inhibition of tumor angiogenesis affords attractive targets for the development of antitumor agents.

A multifaceted approach that targets multiple signaling pathways has been shown to be more effective than the inhibition of a single target.[9–11] The most important consequence of inhibiting multiple RTKs would be to retard tumor resistance by blocking potential 'escape routes'.[12]

Several small molecule inhibitors of RTK targeting the ATP binding site of tyrosine kinases are currently used or are in clinical trials as antitumor agents (Fig. 1).[13,14]

Gangjee et al.[15] designed a series of 2-amino-4-(*m*-bromoanilino)-6-substituted pyrrolo[2,3-*d*]pyrimidines as multiple RTK inhibitors and antiangiogenic agents. A key aspect of this study was the inclusion of a 2-amino group on the pyrrolo[2,3-*d*]pyrimidine scaffold which was anticipated to utilize an additional hydrogen-bond binding site in the Hinge region (ATP binding site) compared to 2-desamino analogs. ATP, which lacks this 2-NH_2 moiety, does not use this site. The RTK inhibitors in the literature, which contain 6–6 and 6–5 bicyclic ring scaffolds usually do not have a 2-amino group to exploit this H-bonding site.

Recently Gangjee et al.[16] reported a series of N^4-(substituted-phenyl)-6-(2-phenylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines (compounds **12**–**18**, Fig. 2) as RTK inhibitors. In this series

Abbreviations: RTK, receptor tyrosine kinase; EGF, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet derived growth factor receptor; CAM, chorioallantoic membrane; ATP, adenine triphosphate.

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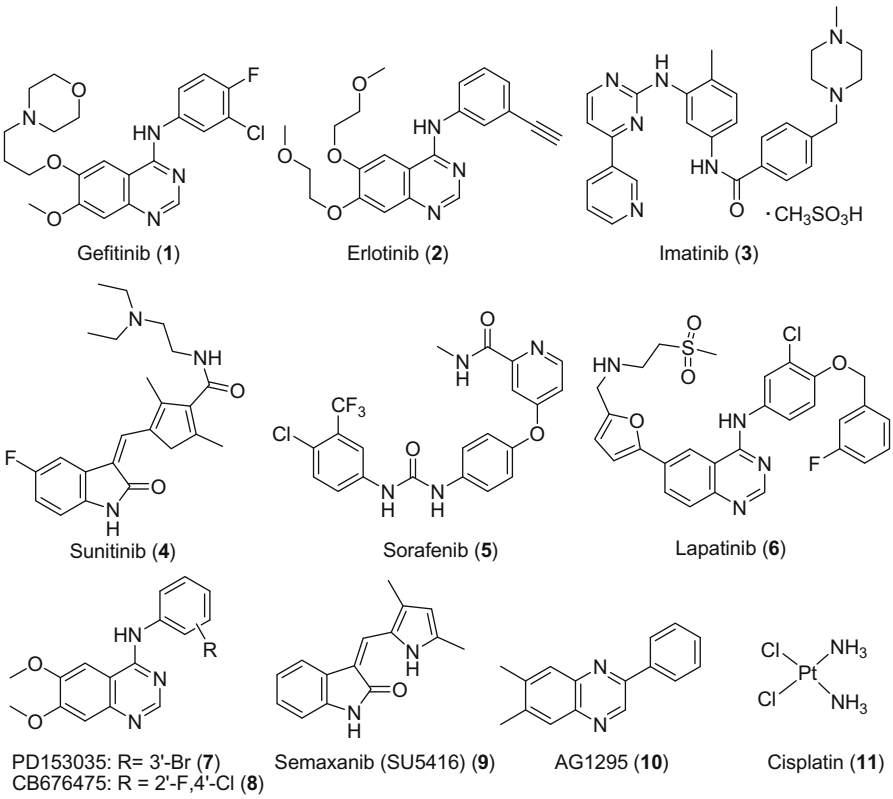
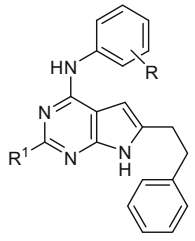


Figure 1. Structures of RTK inhibitors and standards used in the assays.



Comp	R^1	R	Comp	R^1	R
12	NH_2	3'-Br	**19**	H	3'-Br
13	NH_2	3'-ethynyl	**20**	H	3'-ethynyl
14	NH_2	3'-CF_3	**21**	H	3'-CF_3
15	NH_2	3'-Br, 4'-F	**22**	H	3'-Br, 4'-F
16	NH_2	3'-CF_3, 4'-F	**23**	H	3'-CF_3, 4'-F
17	NH_2	2'-F, 4'-Cl	**24**	H	2'-F, 4'-Cl
18	NH_2	3',4'-(C_2H_3N)	**25**	H	3',4'-(C_2H_3N)

12-25

Figure 2. 2-Amino[16] and 2-desaminopyrrolo[2,3-*d*]pyrimidine RTK inhibitors.

the nature and substitutions on the anilino moiety determines both the selectivity and potency against a variety of RTKs in whole cell assays.

It was important to validate our hypothesis regarding the inclusion of a 2-amino group to increase binding and potency of RTK inhibitors.[17] This was especially crucial because most of the marketed tyrosine kinase inhibitors such as **1** (gefitinib), **2** (erlotinib) and **6** (lapatinib) including the substrate ATP lack any substitution at their corresponding 2-position. Hence compounds **19**–**25** (Fig. 2) were designed as 2-desamino analogs of **12**–**18**, in order to compare their activities as RTK inhibitors. Substitutions on the 4-anilino ring were kept same as **12**–**18** from our previous report[16] to allow us for one-to-one comparison between 2-amino analogs (**12**–**18**) and 2-desamino analogs (**19**–**25**).

Superimposition of **12** (Fig. 3) and **19** (Fig. 4) on to ATP from its crystal structure with EGFR (PDB id: 2gs6[19]) using MOE2007.09[18] showed that the pyrrolo[2,3-*d*]pyrimidine ring fits in the same region as the adenine ring of ATP. The *N*-3 and 4-NH moieties of both **12** and **19** are within H-bond distances (≈3 Å) from the backbone NH of Met769 and backbone carbonyl of Gln767, respectively, in the Hinge region of the binding pocket. 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. In addition to this, the 2-NH_2 group of **12** (Fig. 3) is only 2.5 Å away from the backbone carbonyl of Met769 and hence can form an additional H-bond. For the 2-desamino analog **19** (Fig. 4), however, there is no group at the 2-position to provide an H-bond with EGFR. The molecular modeling studies provided further credence to our hypothesis. The distance of the *C*-2 of **19** and backbone carbonyl of Met769 is ≈3.5 Å (Fig. 4). Similarly ATP does not use this H-bonding site. Hence the inclusion of a 2-amino group in our pyrrolo[2,3-*d*]pyrimidine analogs was also expected to perhaps improve selectivity. Thus **19**–**25** were designed so as to compare their biological activities with **12**–**18**.

The synthesis of **19**–**25** is shown in Scheme 1. Intermediate **27**[16] (obtained from phenyl propionic acid in two steps) was treated with the free base of ethoxycarbonylacetamidine hydrochloride **28**[20,21] (obtained from ethyl cyanoacetate) at reflux to afford the pyrrole **29**.[22] Pyrrolo[2,3-*d*]pyrimidine **30** was synthesized from **29** by cyclization with formamide.[23] Chlorination of **30** with phosphorous oxychloride afforded **31**. Nucleophilic displacement of the

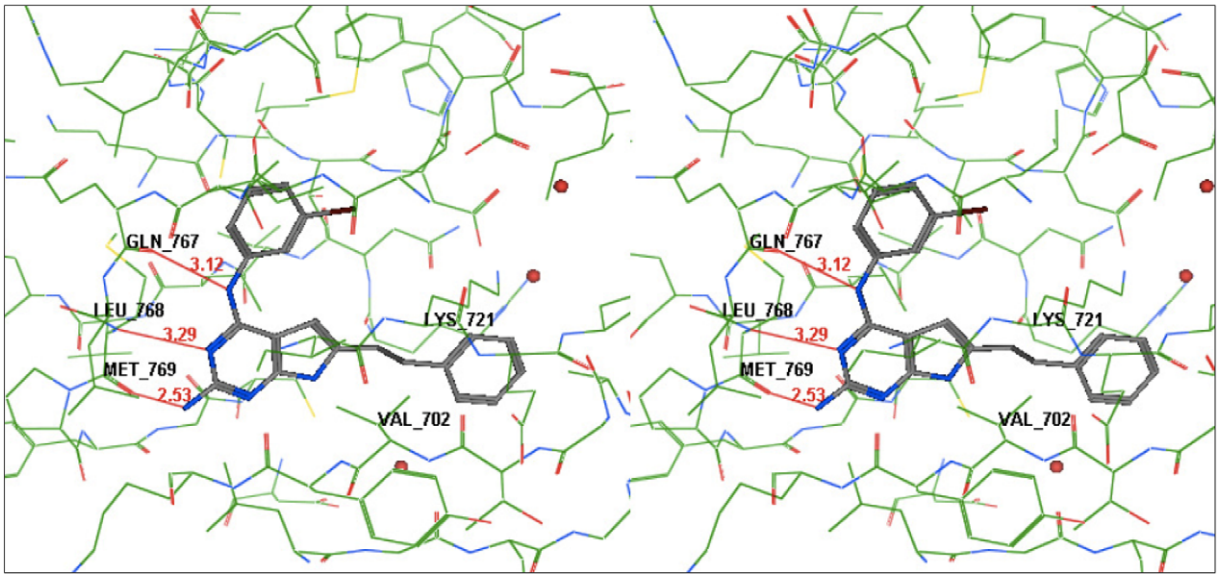


Figure 3. Stereoview of compound **12** in the active site of EGFR [Superimposed on ATP from its crystal structure (pdb id: 2gs6)]; generated using MOE2007.08[18] (Hydrogens removed for clarity).

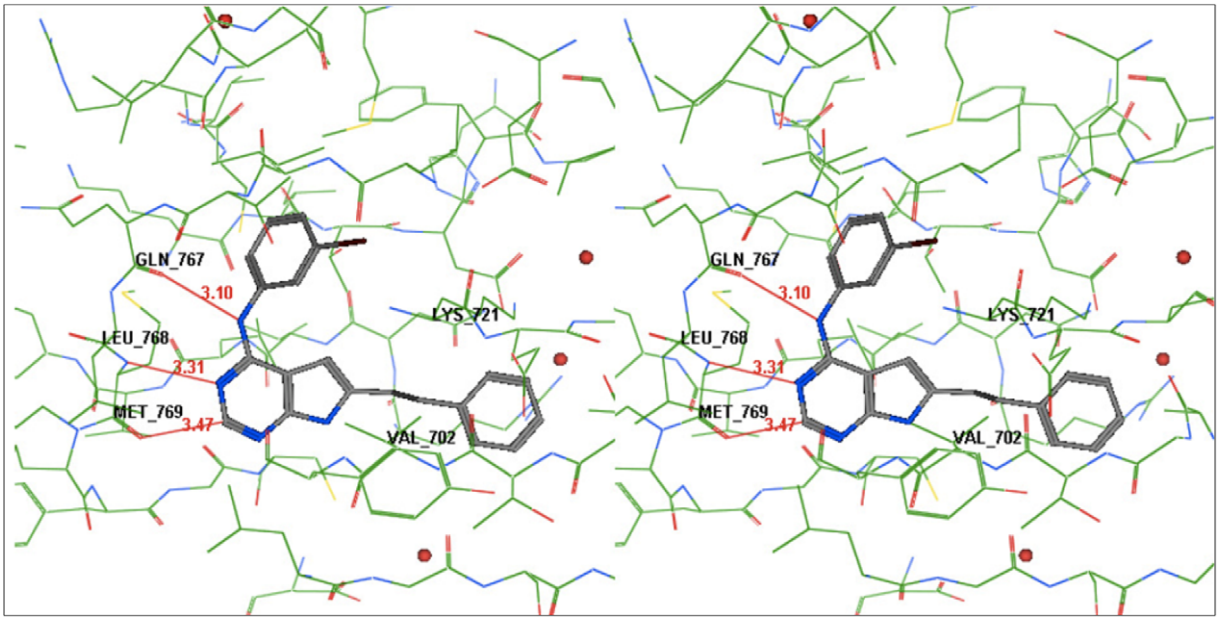
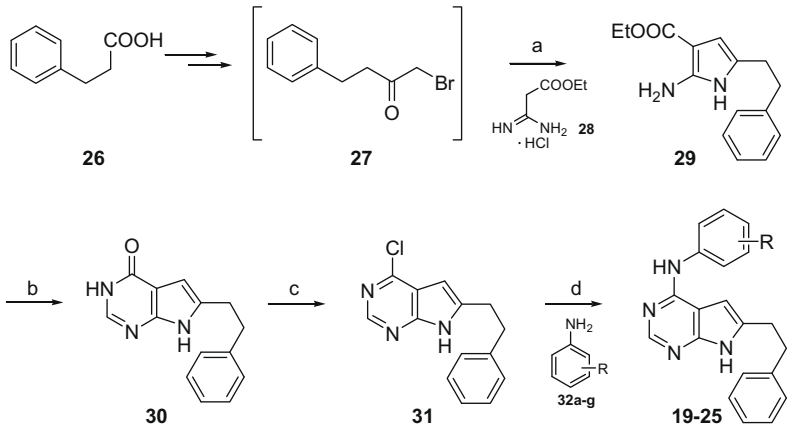


Figure 4. Stereoview of compound **19** in active site of EGFR [Superimposed on ATP from its crystal structure (pdb id: 2gs6)]; generated using MOE2007.08.1[18] (Hydrogens removed for clarity).

4-chloro moiety of **31** with appropriate anilines **32a**–**g** in isopropyl alcohol at reflux for 4 h afforded **19**–**24** (catalytic amounts of conc HCl were added at the start of the reaction) and **25** (no conc HCl needed).

RTK inhibitory activity of the compounds **19**–**25** were evaluated using human tumor cells known to express high levels of EGFR, VEGFR-1, VEGFR-2 and PDFGR-β using a phosphotyrosine ELISA cytoblot[24,25] and are listed in Table 1. Compounds known to inhibit a particular RTK were used as positive controls for these assays. 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 inhibition of the growth of A431 cancer cells known to overexpress EGFR. EGFR has been shown to play a role in the overall survival of the cells.[26] Cell proliferation was assessed using CYQUANT®, a DNA intercalating dye that has been shown to give a linear approximation of cell number[27] and are listed in Table 1. 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[28] and are listed in Table 1. 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 RTK inhibitors vary under different assay conditions, we used a standard (control) compound (Fig. 1) in each of the evaluations. For EGFR the standard was **7** (PD153035); for VEGFR-1 the standard was **8** (CB676475); for



Scheme 1. Synthesis of **19**–**25**. Reagents and conditions: (a) Triethylamine, ethyl acetate, reflux, 25 min; (b) Na, MeOH, formamide, 150 °C, 12 h; (c) phosphorous oxychloride, reflux, 3 h; (d) isopropanol, 2–3 drops concd HCl (except for **25**), reflux.

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

Compd #	R	EGFR inhibition[a]	VEGFR-1 inhibition[a]	VEGFR-2 inhibition[a]	PDGFR-β inhibition[a]	A431 cytotoxicity[a]	CAM angiogenesis inhibition[a]
19	3′-Br	>200	129.4 ± 30.1	112.3 ± 18.1	8.5 ± 2.4	>500	1.93 ± 0.09
(12)		(0.3 ± 0.042)	(45.1 ± 8.9)	(>50)	(11.8 ± 3.7)	(28.6 ± 8.2)	(19.5 ± 5.1)
20	3′-Ethynyl	>200	142.3 ± 23.5	49.4 ± 8.0	>500	>500	5.12 ± 0.46
(13)		(>200)	(>200)	(>200)	(100.4 ± 22.1)	(8.5 ± 1.1)	(1.1 ± 0.07)
21	3′-CF_3	>200	>200	22.1 ± 4.1	>500	>500	5.63 ± 0.67
(14)		>200	(>200)	(>200)	(>500)	(4.6 ± 0.67)	(0.03 ± 0.004)
22	3′-Br, 4′-F	>200	>200	25.2 ± 5.2	192.4 ± 20.8	>500	2.14 ± 0.39
(15)		(22.8 ± 4.7)	(>200)	(132.1 ± 16.7)	(>500)	(6.8 ± 0.71)	(5.9 ± 0.7)
23	3′-CF_3, 4′-F	>200	164.3 ± 18.1	57.5 ± 7.1	>500	>500	19.6 ± 5.9
(16)		(122 ± 30.1)	(>200)	(>200)	(90.0 ± 17.8)	(9.8 ± 1.0)	(0.12 ± 0.12)
24	2′-F, 4′-Cl	>200	>200	40.5 ± 9.3	>500	43.0	36.9 ± 3.2
(17)		(1.32 ± 0.09)	(>200)	(>200)	(>500)	(1.4 ± 0.12)	(8.6 ± 7.6)
25	3′,4′-(C_2H_3N)	56.1 ± 8.7	223.8 ± 41.3	31.6 ± 8.8	104.2 ± 16.7	81.9 ± 10.1	20.1 ± 4.8
(18)		(>200)	(>200)	(24.9 ± 4.1)	(77.4 ± 9.1)	(5.5 ± 1.4)	(50.6 ± 18.8)
7		0.2 ± 0.04				12.6 ± 2.9	
8			17.7 ± 5.5				
9				10.6 ± 2.7		19.2 ± 1.1	0.085 ± 0.0031
10					6.5 ± 1.3		
11						7.65 ± 1.4	

[a] Values in parentheses are for the corresponding 2-amino compounds.[16] All the values are means of three experiments; standard deviation follows the mean value.

VEGFR-2 and the CAM angiogenesis assay the standard was **9** (semaxanib); for PDGFR-β the standard was **10** (AG1295); for the cytotoxicity study against the growth of A431 cells in culture the standard was **11** (cisplatin).

It is important to note that this study involves the use of cellular assays since we believe that the data from such assays can be extrapolated more accurately for the selection of candidates for in vivo studies than isolated kinase inhibitory studies. However, direct comparison of activities of different compounds becomes difficult, since these may involve other factors such as cellular transport and metabolism.

Removal of the 2-amino group was detrimental for the inhibitory activity against EGFR (with the exception of **25**). Compounds **19**–**24** were inactive in the EGFR inhibition assay (IC_{50} >200 μM); while **25** was about 280-fold less active as compared to the standard **7**. In comparison the corresponding 2-amino compounds **12** and **15**–**17** showed significant potency against EGFR. Compounds **19** and **24** showed significant loss of activities as compared to the corresponding 2-amino analogs (**12** and **17**).

Against VEGFR-1, **20** and **23** showed slightly better potencies than the corresponding 2-amino compounds **13**, **16** and **18**; while **19** showed lower VEGFR-1 inhibition than the corresponding 2-amino analog. Compounds **21**, **22** and **24** were inactive against VEGFR-1.

In the VEGFR-2 inhibition assay, compounds **19**–**24** showed improved activity compared to the corresponding 2-amino derivatives (**12**–**17**). Compound **25** was equipotent with the corresponding **18**. Compounds **21**, **22** and **25** were only 2–3-fold less potent than the standard **9**. All the compounds showed 2-digit micromolar inhibition against VEGFR-2 except **19**; which was only 10-fold less potent than the standard **9**.

In the PDGFR-β inhibition assay, **20** and **23** were inactive; while corresponding 2-amino compounds **13** and **16** exhibited 2- to 3-digit micromolar inhibition against PDGFR-β. Additionally compounds **21** and **24** were inactive. Compound **19** was equipotent to the corresponding 2-amino analog **12**; while **22** was more potent than its corresponding 2-amino analog **15**.

In the A431 cytotoxicity assay all of the 2-desamino compounds **19**–**25** showed diminished potency compared to the corresponding 2-amino analogs **12**–**18**. Compounds **19**–**23** were inactive in this assay. Compound **24** was the best compound in the 2-desamino series; however it was about 300-fold less active than the corresponding 2-amino compound **17** and sixfold less active than the standard **11**.

In the CAM angiogenesis inhibitory assay all the 2-desamino compounds showed one to two-digit micromolar inhibition of angiogenesis and were significantly less active than the standard **9**. Compounds **20**, **21**, **23** and **24** were significantly less potent than corresponding 2-amino analogs (**13**, **14**, **16** and **17**). The biggest difference was seen for **21** (186-fold less potent than **14**) and for **23** (163-fold less potent than **17**). Compounds **19**, **22** and **25** were better in potency than corresponding 2-amino analogs (**12**, **15** and **18**).

In summary, removal of the 2-amino group in N^4-(substituted-phenyl)-6-(2-phenylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-2,4-diamines led to a significant loss of activity against EGFR (except **25**), PDGFR-β (except **19** and **22**) and in the A431 cytotoxicity assay (in all seven cases). Interestingly, removal of the 2-amino group was conducive for the inhibition of VEGFR-2, (in five of seven cases). In the CAM angiogenesis assay, however, the 2-desamino compounds (**19**–**25**) showed only one to two-digit digit micromolar inhibition whereas the 2-amino analogs (**14** and **16**) showed nanomolar inhibition[16] lending credence to our hypothesis[15] of the importance of the 2-amino group for antiangiogenic activity. The results of this study also indicate that the inclusion a 2-amino group in the marketed RTK inhibitors such as **1**, **2** and **6** may improve their potency and perhaps their activity against multiple RTKs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.064.

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