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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2776–2780
Bioorganic & Medicinal Chemistry Letters

Inhibitors of the tyrosine kinase EphB4. Part 1: Structure-based design and optimization of a series of 2,4-*bis*-anilinopyrimidines

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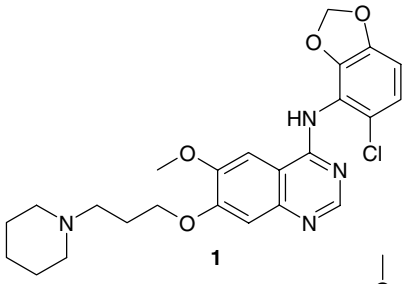
Received 25 March 2008; revised 4 April 2008; accepted 6 April 2008
Available online 10 April 2008

Abstract—A series of *bis*-anilinopyrimidines have been identified as potent inhibitors of the tyrosine kinase EphB4. Structural information from two alternative series identified from screening efforts was combined to identify the initial leads.

The erythropoietin-producing hepatoma amplified sequence (Eph) family is the largest known family of receptor tyrosine kinases, with 14 receptors and 8 cognate ephrin ligands identified.[1] There is growing evidence that Eph receptor signalling may contribute to tumorigenesis in a wide variety of human cancers, either on tumour cells directly, or indirectly via modulation of vascularisation. Many Eph receptors are over-expressed in various tumour types[2,3] and expression of EphB4 is up-regulated in tumours such as neuroblastomas, leukaemia's, breast, liver, lung and colon. Moreover, various in vitro and in vivo studies particularly regarding EphB4 have indicated that over-expression of Eph receptors on cancer cells is able to confer tumourigenic phenotypes such as proliferation and invasion, consistent with the speculated role in oncogenesis.

Inhibition of EphB4 expression using interfering-RNA or antisense oligodeoxynucleotides inhibited proliferation, survival and invasion of PC3 prostate cancer cells in vitro and in vivo.[4] In addition, there is good evidence that EphB4 may contribute to tumour vascularisation.[5,6] Members of the Eph family including EphB4 are expressed on endothelial cells and transgenic studies have shown that disruption of EphB4[7] or its ligand ephrinB2[8] causes embryonic lethality associated with vascular modelling defects consistent with a critical role in vessel development. EphB4 activation also stimulates endothelial cell proliferation and migration in vitro[9] and inhibition of EphB4 signalling using soluble extracellular domains of EphB4 has been shown to inhibit tumour growth and angiogenesis in in vivo xenograft studies.[10,11]



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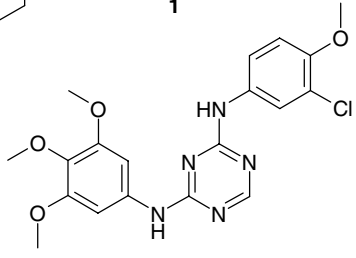

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Keywords: Kinase; EphB4; Structure-based design; Lead identification.
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doi:10.1016/j.bmcl.2008.04.015

In our search for inhibitors of EphB4 tyrosine kinase, preliminary screening[12] of libraries of kinase inhibitors highlighted compound **1**, a known anilinoquinazoline inhibitor of the c-Src tyrosine kinase from in-house research[13] as a potent inhibitor, and also **2**, a modestly potent *bis*-anilinotriazine, analogues of which have previously been reported as kinase inhibitors.[14] Determination of the binding modes of these two inhibitors was achieved by soaking into the catalytic domain of EphB4 (D598-G892 with a Y774E mutation)[15] and revealed as expected that both occupied the ATP-binding site. Compound **1** forms a key hydrogen bond between the quinazoline N-1 and Met696 (Fig. 1(a)), while compound **2** presents a donor–acceptor pair to Met696 (Fig. 1(b)). Intriguingly, protein overlay of these two structures (Fig. 1(c)) highlighted exact convergence of the heterocyclic hinge-binding groups, with the different C-4 anilino groups opposed. The dioxole aniline of the more potent **1** is buried in the selectivity pocket, the hydrophobic pocket beyond the Thr693 gatekeeper residue, whereas the aniline of **2** points towards the ribose binding site,[16] suggesting that a hybrid of the aniline of **1** and the core of **2** may also result in potent inhibition of EphB4.

In the event, we elected to examine both triazine and pyrimidine core hybrids. Synthesis of these analogues is outlined in Scheme 1, and is representative of the synthesis of related analogues prepared in the course of this study. Accordingly, both 2,4- and 4,6-dichloropyrimidine, and 2,4-dichlorotriazine **3** were reacted with 5-chlorobenzo[1,3]dioxol-4-amine to give intermediates **4**, which in turn were reacted with 3,4,5-trimethoxyaniline at elevated temperatures to give the target regioisomeric pyrimidines **6** and **7**, and triazine **5**.

The compounds listed in Table 1 were evaluated in an EphB4 kinase assay measuring the inhibition of phosphorylation of a synthetic peptide substrate at K_m ATP concentration.[17] The dioxole-containing triazine **5** and 4,6-pyrimidine isomer **6** both showed a modest improvement in activity compared with hit **2**, although we did not have structural confirmation that this was due to any shift in the aniline orientation. Gratifyingly, 2,4-pyrimidine analogue **7** showed potent activity at a level comparable to quinazoline hit **1**, and Figure 1(d) shows the structure of this compound bound to the catalytic domain of EphB4 confirming indeed that the C-4 aniline has re-orientated towards the selectivity pocket

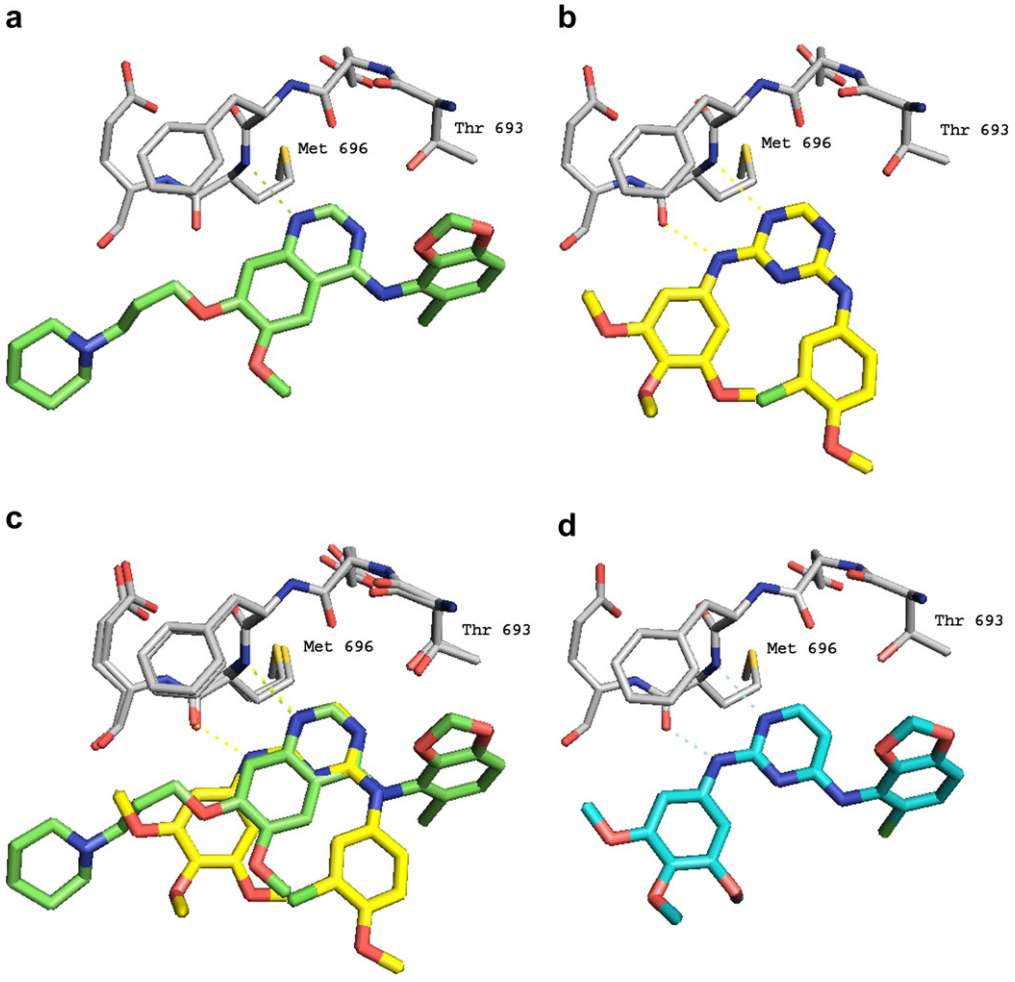
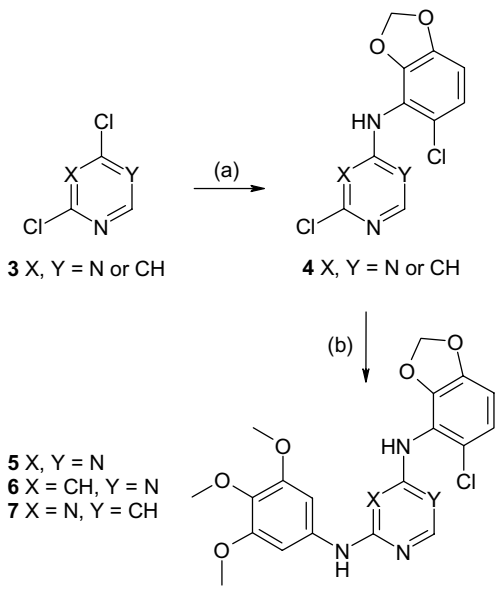


Figure 1. (a) Structure of anilinoquinazoline **1** bound to the catalytic domain of EphB4. (b) Structure of *bis*-anilinotriazine **2** bound to the catalytic domain of EphB4. (c) Overlay of the structures of **1** and **2** obtained through alignment of protein structure. (d) Structure of *bis*-anilinopyrimidine **7**, prepared on the basis of this alignment, bound to the catalytic domain of EphB4.



Scheme 1. Synthesis of *bis*-anilinotriazines and pyrimidines. Reagents and conditions: (a) 5-chlorobenzo[1,3]dioxol-4-amine,[13] NaH, DMA, 0–25 °C; (b) 3,4,5-trimethoxyaniline, HCl in Et_2O, *n*-BuOH, DMA, 120 °C.

Table 1. EphB4 inhibition data for compounds **1**, **2** and **5**–**7** and conformational energies and computed hydrogen bond acceptor abilities of the different cores

Entry	Core	EphB4 IC_{50} (μM)[a]	Relative energy of bound conformation and global minimum (kcal/mol)	Calculated $\log K_\beta$ or N-1
1	Quinazoline	0.35 ± 0.13	0.7	2.65
2	Triazine	16	—	—
5	Triazine	2.96 ± 0.71	1.5	1.42
6	4,6-Pyrimidine	2.54 ± 0.35	2.8	1.72
7	2,4-Pyrimidine	0.24 ± 0.16	0.9	2.12

[a] For determinations where $n \geqslant 2$, standard deviation is given.

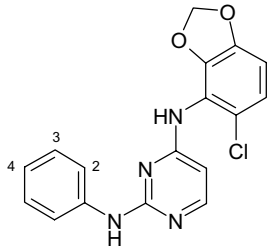
as hypothesised. It is interesting to note that this most active of the hybrids has a CH group at C-5 which in both **1** and **2** is N.

The combined effect of the hydrogen bonding ability of the different cores and their innate conformational preferences can rationalize these observations. The crystal structures of both **1** and **7** reveal that the dioxole ring sits in the selectivity pocket and consequently demands a conformation of the aniline ring whose energy is reasonably close to the global minimum energy of the ligand. A further conformational restriction applies to the pyrimidine and triazine cores because the region adjacent to the hinge in which they form their principal interactions requires a relatively flat ligand, such as that found for quinazoline **1** or indeed the adenine of ATP. The ligand-bound structures reveal the dihedral angles that are preferred in the protein complex, and when these are applied as constraints in quantum mechanical calculations, the energies relative to presumed global minima are given in Table 1.[18]

When the ligands are constrained to the conformation required for binding, the hydrogen bonding acceptor ability ($\log K_\beta$ values)[19] of the nitrogen that forms the primary interaction with the hinge backbone can be estimated by calculation.[20] These calculations indicate that quinazoline **1** and 2,4-pyrimidine **7** both have conformational energies which are closer to the global minimum, and demonstrate the most effective hydrogen bonding capability when compared to triazine **5** and 4,6-pyrimidine **6**, and this would appear to be reflected in the experimentally observed enzyme data. It is noteworthy that 4,6-pyrimidine **6** has a conformational energy above the global minimum that is approximately the same as that calculated for the more active 2,4-pyrimidine **7** (both 0.8 kcal/mol), that is until the anilines at the 6-position (for **6**) and 2-position (in the case of **7**) are constrained to the observed dihedral angles. This produces steric repulsion in the 4,6-pyrimidine that is not present in the 2,4-pyrimidine due to the adjacent nitrogen in the pyrimidine core. The hydrogen bonding ability of N-1 is also dependent on this conformation and before the constraint is imposed, the 2,4- and 4,6-isomers have rather similar $\log K_\beta$ values (2.05 and 2.14, respectively).

Since we considered that the trimethoxyaniline represented a potential DMPK liability, we next elected to examine alternative anilines at the C-2 position, and in particular the electronic, steric and positional effects of various substituents. Table 2 highlights the systematic examination of these factors, whilst keeping the C-4 aniline fixed. As expected, the three methoxy substituents in **7** showed a key contribution to the observed potency, with the simple aniline analogue **8** showing a 6-fold drop in activity. All of the substituents examined when placed in the 2-position resulted in loss of activity relative to **8**. This effect was particularly dramatic for electron-withdrawing substituents such as cyano, sulfonamide and sulfone (compounds **21**, **24** and **27**) but was observed for simple substituents such as F and methoxy (compounds **9** and **18**). It is clear from the crystal structures in Figure 1((b) and (d)) that preference exists for the C-2 aniline to be co-planar with the central heterocyclic ring, allowing the key NH-donor to be presented to the hinge region in the correct orientation whilst allowing optimal electron delocalisation in the ligand itself, and any ortho-substitution might be expected to adversely impact this. When comparing triads of the same substituent across 2-, 3- and 4-positions it is evident that potency is significantly enhanced at the 3-position, with the activity order $3 < 4 \ll 2$ maintained for all substituents (compare **9**–**11**, **12**–**14**, etc.). All the 3-substituents examined showed enhancements in activity relative to unsubstituted parent **8**, although the most potent activity was observed for the bulky, strongly electron-withdrawing sulfonamide (**25**) and sulfone (**28**) groups. Importantly, these demonstrated potency improvements over initial hybrid **7**, indicating that a strongly electron-rich C-2 aniline is not a requirement for activity. In addition, a range of both large and small, electron-donating and -withdrawing substituents furnished submicromolar activity at this position including methyl (**16**), methoxy (**19**), cyano (**22**) and morpholinyl (**31**).

Table 2. EphB4 inhibition data for C-2 aniline variants



Substituent	EphB4 IC_{50} [a] (μM)					
	Entry	2-	Entry	3-	Entry	4-
H			**8**	1.55 ± 0.07		
F	**9**	5.00 ± 0.28	**10**	1.15 ± 0.07	**11**	2.30 ± 0.10
Cl	**12**	11.32 ± 0.58	**13**	1.33 ± 0.79	**14**	6.00 ± 1.00
Me	**15**	13.96 ± 1.41	**16**	0.46 ± 0.04	**17**	1.60 ± 0.00
MeO	**18**	3.88 ± 0.57	**19**	0.64 ± 0.07	**20**	1.94 ± 0.21
CN	**21**	20.49 ± 0.71	**22**	0.55 ± 0.15	**23**	1.25 ± 0.07
SO_2NH_2	**24**	35.50 ± 0.71	**25**	0.04 ± 0.02	**26**	0.30 ± 0.12
SO_2Me	**27**	41.00 ± 0.00	**28**	0.09 ± 0.05	**29**	0.63 ± 0.01
1-Morpholinyl	**30**	7.85 ± 3.47	**31**	0.58 ± 0.12	**32**	1.00 ± 0.00

[a] For determinations where $n \geqslant 2$, standard deviation is given.

Examination of substituent effects at the 4-position indicated these inhibitors to be less effective than those with the same substituents at the 3-position, generally at least 2-fold but greater for some examples. It is interesting to note that the most potent substituents in the 4-position mirror those in the 3-position, namely the sulfonamide (**26**) and sulfone (**29**) both show sub-micromolar activity comparable with lead **7**. Halogen substituents (F, Cl) were seen to be either poorly tolerated or offer no benefit over the unsubstituted parent in all positions (compounds **9**–**11** and **12**–**14**).

On the basis of these data we chose to examine the 3-sulfone **28** in greater detail. This compound was examined in a cellular assay of EphB4 inhibition,[21] and was found to inhibit phosphorylation with an IC50 of 0.19 ± 0.07 μM, in good agreement with its enzyme inhibition value. In a panel of kinase assays, **28** was shown to be remarkably selective. Table 3 highlights the data from this selectivity panel. Compound **28** is inactive or weakly active against most of the kinases tested. Only the EphB2 and Src enzymes are potently inhibited, and this is expected both on the basis of high sequence homology to EphB4 (85% and 44%, respectively, in the kinase domains) and the presence of a C-4 aniline previously optimised for potent Src inhibition in the quinazoline series.[13] Table 4 summarises selected physicochemical and pharmacokinetic parameters in the rat for compound **28**. log *D* is acceptable, and solubility is modest with a reasonably high degree of protein binding, although not prohibitively so, as a lead for further optimisation. The in vivo profile in rat is also encouraging, showing a modest but acceptable bioavailability of 30%, and low clearance, potentially indicating solubility limited absorption.

In summary, we have used structural information from two distinct series bound in the active site of EphB4 to

Table 3. Selectivity data for compound **28**

Kinase	IC_{50} (μM)
EphB2	0.05
Src	<0.10
CSK	10.7
CDK2	17.6
FAK	19.7
JAK3	20.5
p38	27.1
IGF	>30
ZAP70	>30
EGF	>30
KDR	>30
FGF	>30
PLK	>30
CHK1	>30
JNK	>30
PKA	>30

Table 4. Selected physicochemical and rat pharmacokinetic data[a] for compound **28**

log *D* (pH 7.4)	Solubility[b] (μM)	Rat PPB% free drug[c]	Bioavailability (%)	Cl (ml/min/kg)	V_{dss} (L/kg)	$t_{1/2}$ (po) (h)	AUC (po)[d] (μM h)
3.13	30	0.74	30	6.1	0.5	5.3	4.0

[a] From an oral dose of 5 μmol/kg, iv dose of 0.5 μmol/kg in Han Wistar rats.
[b] Aqueous solubility measured in pH 7.4 buffer on pure but generally amorphous material.
[c] Measured at 37 °C.
[d] Total AUC at the oral dose administered.

generate a novel series of 2,4-*bis*-anilinopyrimidine inhibitors. SAR studies around the C-2 hinge-binding aniline indicate a strong preference for meta-substitution, particularly with electron-withdrawing groups. One example from this work, **28**, has shown an encouraging kinase selectivity and pharmacokinetic profile and serves as the basis for further hit to lead optimisation. Further optimisation of, in particular the C-2 aniline of this class of inhibitors is described in the subsequent communication.

Acknowledgment

We thank Jonathan Wingfield for technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.015.

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21. CHO–K1 cells were engineered to stably express an EphB4-Myc-His construct. The endpoint assay used a sandwich ELISA to detect EphB4 phosphorylation status. Myc-tagged EphB4 from treated cell lysate were captured via an anti-c-Myc antibody and the phosphorylation status of captured EphB4 was then measured using a generic phosphotyrosine antibody. For further details see Ref. 17.