Bioorganic & Medicinal Chemistry Letters 18 (2008) 5717–5721

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Inhibitors of the tyrosine kinase EphB4. Part 2: Structure-based discovery and optimisation of 3,5-*bis* substituted anilinopyrimidines

Catherine Bardelle, Tanya Coleman, Darren Cross, Sara Davenport, Jason G. Kettle *, Eun Jung Ko, Andrew G. Leach, Andrew Mortlock, Jon Read, Nicola J. Roberts, Peter Robins, Emma J. Williams

AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

ARTICLE INFO

Article history:
Received 4 September 2008
Revised 23 September 2008
Accepted 24 September 2008
Available online 27 September 2008

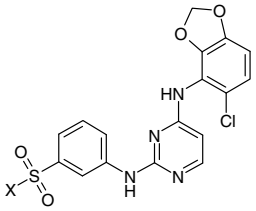
Keywords:
EphB4
Tyrosine kinase
Structure-based design
Pyrimidine

ABSTRACT

Crystallographic studies of a range of 3-substituted anilinopyrimidine inhibitors of EphB4 have highlighted two alternative C-2 aniline conformations and this discovery has been exploited in the design of a highly potent series of 3,5-disubstituted anilinopyrimidines. The observed range of cellular activities has been rationalised on the basis of physicochemical and structural characteristics.

The field of kinase inhibition has seen an explosion in activity over the last ten years, and much of this has been directed towards inhibition of the receptor tyrosine kinases known to play a pivotal role in the aberrant signalling that is characteristic of the uncontrolled proliferation of tumours. The erythropoietin-producing hepatoma amplified sequence (Eph) family is the largest known group of such kinases,[1] and together with their ephrin ligands are increasingly implicated in tumourigenesis in a wide variety of human cancers, either on tumour cells directly, or indirectly via modulation of vascularisation.[2–4] In the preceding paper[5] which referred to the first EphB4 crystal structures,[6] we outlined the discovery of a novel series of potent and selective 2,4-*bis* anilinopyrimidines as inhibitors of EphB4 derived from structural overlays of two alternative inhibitor series bound in the active site.[7] Initial chemical optimisation led to the observation of a strong preference for a 3-substituted anilino C-2 hinge-binding group, with strong electron-withdrawing groups such as sulfonamide **1** and sulfone **2** most preferred. Substitution at the ortho position in this ring was generally poorly tolerated, and all substituents at para were less active than the same groups at the meta position.

In order to try and understand these preferences further, the crystal structure of sulfonamide ligand **1** in complex with EphB4 was determined.[8] The inhibitor adopts the expected conformation, with the benzdioxole moiety buried in the selectivity pocket, and a donor–acceptor hydrogen bond to the hinge region at Met696, mimicking the binding of ATP (Fig. 1a and b). Intriguingly, however, the C-2 aniline of **1** is able to adopt a dual conformation within the protein structure, with the sulfonamide alternatively hydrogen bonding towards the hinge and Glu697, or back towards the glycine-rich loop and Ile621 (Fig. 1a). The electron density in Figure 1a suggests the two binding modes are approximately equally populated (each has been modelled with an occupancy of 0.5, where 1.0 is full occupancy). A view along the hinge (Fig. 1b) demonstrates the proximity of the hydrogen bonds to the protein, and the near overlap of the C-2 phenyl rings in the differing binding modes. The initial SAR studies highlighted a strong preference for groups at this position that were capable either of accepting or donating a hydrogen bond (and in the case of **1**, both simultaneously) and the determined structure of **1** may go some way to explain this observation. A second striking feature of the structure of **1** is that there appears to be no movement in the protein to accommodate these alternative orientations, opening up the possibility that *bis*-meta, that is, 3,5-disubstituted anilines would be tolerated at C-2 of the pyrimidine, and could potentially lead to enhancements in potency.



1 X = NH_2
2 X = CH3

* Corresponding author. Tel.: +44 1625517920; fax: +44 1625586707.
E-mail address: jason.kettle@astrazeneca.com (J.G. Kettle).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.09.087

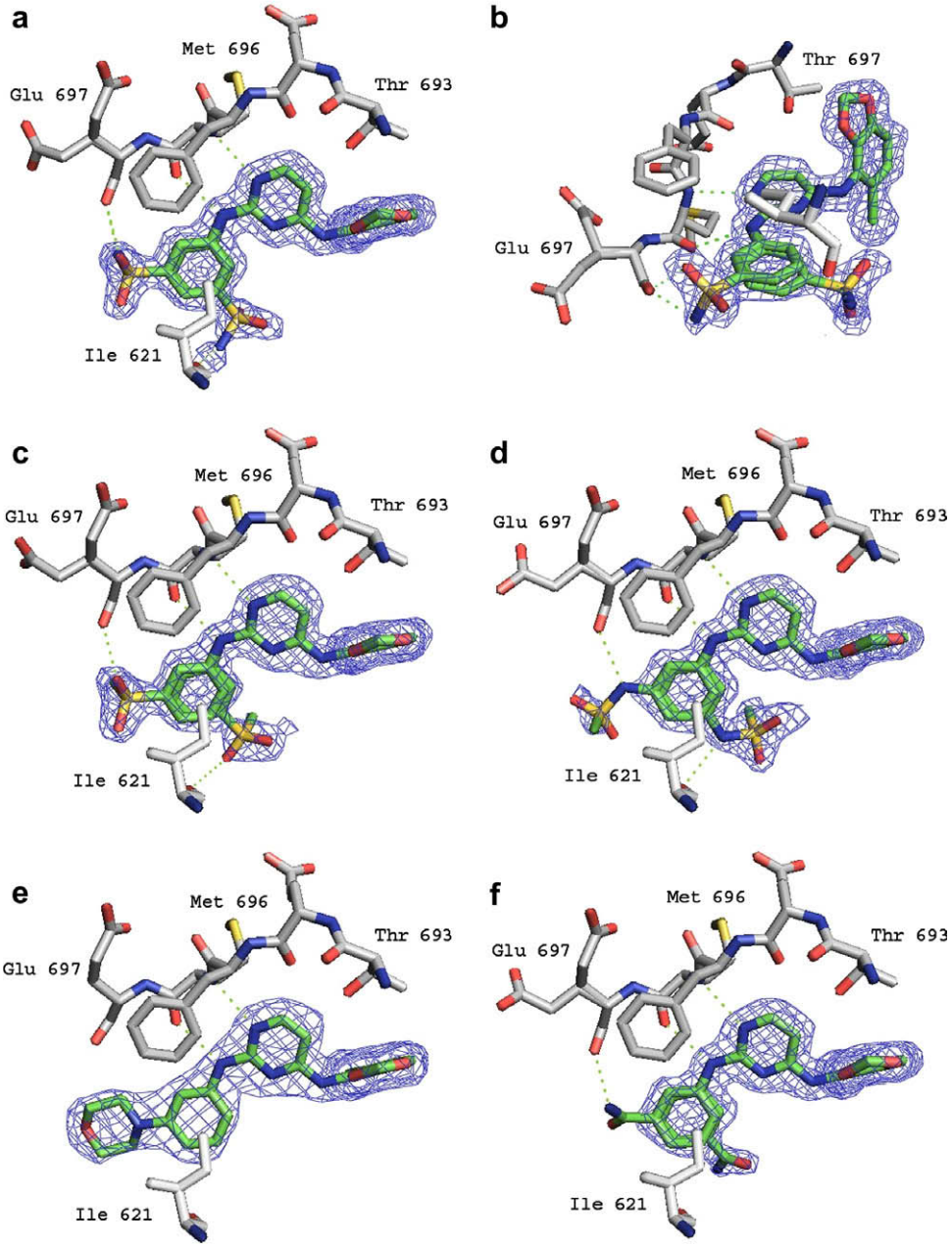
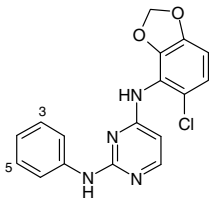


Figure 1. Binding of 3-substituted anilinopyrimidines to EphB4. For clarity, final $2F_o - F_c$ electron density contoured at 1σ is drawn around the ligand only. (a) Binding of sulfonamide **1** to the catalytic domain of EphB4 showing the dual conformation adopted by the C-2 anilino group. (b) An alternative view of bound **1** along the hinge-axis showing the sulfonamide hydrogen-bonding both towards the hinge and Glu697 and towards the glycine-rich loop and Ile621. (c) Structure of sulfone **2** shows an occupancy ratio of approximately 0.7–0.3 (Glu697/Ile621). (d) Structure of aminosulfonamide **3** shows occupancy ratio of approximately 0.3–0.7 (Glu697/Ile621). (e) Structure of morpholine **4** shows full occupancy of the binding mode oriented towards Glu697. (f) Structure of primary carboxamide **5** shows almost full occupancy of the binding mode oriented towards Ile621.

Prior to investigation of which 3,5-disubstituted anilines might be examined, two questions remained unanswered. Firstly, which of the possible different potent meta-substituents identified from the initial SAR studies would be optimal in combination, and secondly which other of these substituents, if any, demonstrated propensity for a dual binding mode? At the outset it was reasonably expected that combination of a meta-substituent with preference for one binding mode would not be enhanced by a second meta-substituent known to prefer the same binding orientation. In an attempt to understand which substituents might be thus combined we obtained crystal structures of compounds **2**–**5** (Table 1) bound to EphB4,[9] and it is clear that the nature of the 3-substituent is key to determining the orientation of the C-2 aniline within the active site. Similar to **1**, the closely related sulfone **2** showed a dual binding mode, with a small preference for binding towards Glu697 (occupancy 0.7)[10] over Ile621 (occupancy 0.3; Fig. 1c). Conversely the aminosulfonamide compound **3** demonstrated the opposite preference in favour of Ile621 (occupancy 0.8; Fig. 1d). The morpholino-substituted compound **4** showed exclusive binding oriented towards Glu697 (Fig. 1e), and conversely the electron density for the primary carboxamide **5** showed near-exclusive binding towards Ile621 (Fig. 1f; occupancy modelled as >0.8). On the basis of these observations, it was tempting to speculate which might be the optimal combinations, for example complementary

Table 1
EphB4 inhibition data for 3-substituted and 3,5-disubstituted anilinopyrimidines

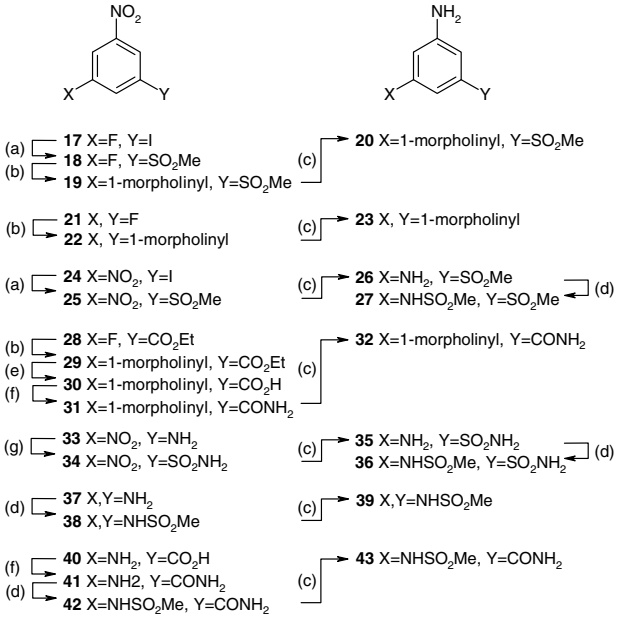


Compound	3-	5-	EphB4 IC_{50}[a] (μM)	Cell IC_{50} (μM)	Binding to Glu697[b] (%)	Binding to Ile621[b] (%)
1	$-SO_2NH_2$	H	0.040 ± 0.020	0.172 ± 0.073	50	50
2	$-SO_2Me$	H	0.090 ± 0.050	0.190 ± 0.070	70	30
3	$-NHSO_2Me$	H	0.794 ± 0.226	0.741	30	70
4	1-Morpholinyl	H	0.580 ± 0.120	NT	100	0
5	$-CONH_2$	H	0.220 ± 0.014	0.900 ± 0.100	<20	>80
6	$-SO_2Me$	$-SO_2Me$	0.114 ± 0.095	0.209		
7	$-SO_2Me$	$-NHSO_2Me$	0.012 ± 0.002	2.054 ± 0.268		
8	$-SO_2Me$	$-CONH_2$	0.023 ± 0.001	22.200		
9	$-SO_2Me$	1-Morpholinyl	0.002 ± 0.002	0.032 ± 0.02		
10	$-NHSO_2Me$	$-SO_2NH_2$	0.001 ± 0.001	14.117 ± 6.095		
11	$-NHSO_2Me$	1-Morpholinyl	0.002 ± 0.002	0.170		
12	$-NHSO_2Me$	$-NHSO_2Me$	0.015 ± 0.004	7.175		
13	$-NHSO_2Me$	$-CONH_2$	0.370 ± 0.014	>30		
14	1-Morpholinyl	1-Morpholinyl	0.002 ± 0.004	0.017 ± 0.011		
15	1-Morpholinyl	$-CONH_2$	0.013 ± 0.001	0.887 ± 0.271		
16	$-CONH_2$	$-CONH_2$	0.077 ± 0.007	>30		

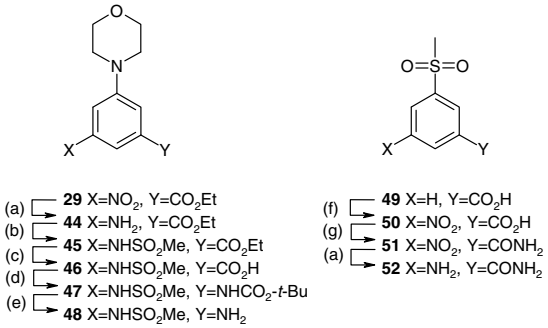
[a] For determinations where $n \geqslant 2$, standard deviation is given.
[b] Binding percentage is estimated on the basis of the cage electron densities of the structures shown in Figure 2 and is approximate.

substituents such as sulfone (as in **2**) with aminosulfonamide (as in **3**) or morpholinyl (as in **4**) with carboxamide (as in **5**) might be expected to give enhancements in potency through reinforcement of the individual substituents preferred binding orientations.[11]

In the event, we synthesized as many of the combinations of these five substituents as could be accessed in a reasonable timeframe, and these combinations are highlighted in Table 1. Synthesis of the requisite 3,5-disubstituted anilines is detailed in Schemes 1 and 2 and the final inhibitors were assembled as previously described.[5,12] Synthesis of the anilines was initiated for the most part from commercially available 3,5-disubstituted nitrobenzenes and involved a core group of reactions whose order was varied to install each of the substituents as required. Thus in each case, morpholine was introduced by reaction with a fluorobenzene activated by a meta-nitro group[13] (see anilines **20**, **23**, **32** and **48** used to give target compounds **9**, **14**, **15** and **11**, respectively), and methanesulfone was installed by copper-catalysed coupling with methanesulfinate anion on the requisite iodobenzene[14] (see anilines **20** and **27** used in inhibitors **9** and **7**, respectively). Aminosulfonamides such as those present in inhibitors **7**, and **10**–**13** were introduced by simple methanesulfonylation of the pre-cursor anilines (see anilines **27**, **36**, **39**, **43** and **48**) and primary carboxamides (as in inhibitors **8**, **13**, **15** and **16**) accessed via HATU coupling of



Scheme 1. Synthesis of anilines used in inhibitors **7**, **9**–**10**, **12**–**15**. Reagents and conditions: (a) $MeSO_2Na$, CuI, DMF, 110 °C; (b) Morpholine, DMSO, 100 °C; (c) H_2, 10% Pd/C, EtOH; (d) $MeSO_2Cl$, pyridine, DCM, 25 °C; (e) aq NaOH, MeOH, THF, 25 °C; (f) NH_4Cl, HATU, DIPEA, DMF, 25 °C; (g) i—$NaNO_2$, concd HCl, water, CuCl, SO_2; then ii—NH_3, MeOH.



Scheme 2. Synthesis of anilines used in inhibitors **8** and **11**. Reagents and conditions: (a) H_2, 10% Pd/C, EtOH; (b) $MeSO_2Cl$, pyridine, DCM, 25 °C; (c) aq NaOH, MeOH, THF, 25 °C; (d) DPPA, DIPEA, *t*-BuOH; (e) concd HCl, MeOH, 70 °C; (f) concd HNO_3, concd H_2SO_4, 85 °C; (g) NH_4Cl, HATU, DIPEA, DMF, 25 °C.

the appropriate benzoic acid derivatives (see anilines **32**, **43** and **52**). For aniline **48** (Scheme 2, used in inhibitor **11**) the aniline moiety was introduced via Curtius rearrangement of acid **46**. Aniline **52** (present in inhibitor **8**) was introduced via reduction of a nitro group installed via nitration of commercially available sulfone acid **49**.

Introduction of a second sulfone group to **2** to give *bis*-sulfone **6** led to comparable enzyme activity[15] but was clearly not detrimental. When the sulfone was combined with a second aminosulfonamide group as in **7**, an increase in enzyme activity to 0.012 μM was observed, a figure significantly more active than the contribution from each substituent on its own (in **2** and **3**), and appears to lend support to the strategy for combining substituents with complementary binding modes. Similarly compound **8**, comprising a sulfone favouring binding towards Glu697, and a primary carboxamide favouring binding to Ile621 resulted in an inhibitor significantly more potent than either substituent alone (compare to **2** and **5**). Other examples of the synergistic nature of specific combinations can be found in compounds **11** (combining a morpholinyl and aminosulfonamide to give a 0.002 μM inhibitor) and **15** (combining morpholinyl with carboxamide to give a 0.013 μM inhibitor) both of whose C-2 anilines might be expected to be potent in combination, from the structural studies outlined above. Similarly the one group for which there was no overall binding preference, the sulfonamide (as in **1**) also shows potent activity in the single combination examined (here with aminosulfonamide as in **10**) at 0.001 μM.

Surprisingly, it was observed that many other combinations also led to potent inhibition, including combinations which singly at least, were shown to prefer the same binding orientation. The most striking example of this is *bis*-morpholine **14** that in **4** favours a single conformation, but in combination leads to a very potent inhibitor, at 0.002 μM nearly 300-fold more active than with a single substituent. It is noteworthy that this substantial potency increase occurs in tandem with a 0.5 log unit decrease in *Clog P* relative to **4**, following introduction of a second morpholine. Morpholine in combination with a sulfone (as in **9**) was highly active despite both substituents independently showing a favoured binding towards Glu697 and similarly *bis*-aminosulfonamide **12** and *bis*-carboxamide **16** were also very potent. The inhibitor with carboxamide and aminosulfonamide groups, **13**, showed enzyme activity intermediate between the contributions from each substituent in isolation (**3** and **5**). The majority of 3,5-disubstituted anilines examined are not only tolerated, but positively enhance enzyme inhibition potency. Despite our initial assumption that only substituents with complementary binding modes might be expected to enhance potency, it is clear from the data that two meta-substituents are favoured over one, and the observation that the global protein structure does not change to accommodate the different aniline orientations may in part explain this.

In comparison with the enzyme data, the activity in a cellular assay of EphB4 inhibition[16] for these compounds highlights a much broader spread of data, ranging from potent inhibition for **9** and **14** to activity higher than the top concentration tested for **13** and **16**. Analysis of the correlation of the difference in enzyme to cell activity and parameters such as number of hydrogen-bond donors and lipophilicity (*Clog P*) is informative (Fig. 2). The cell activity is seen to drop off rapidly as a function of increasing number of hydrogen-bond donors or decreasing *Clog P*.[17] These data may be indicative of a problem of permeability (since adequate lipophilicity is required for permeation and higher numbers of donors may impair permeability across the cell membrane) or efflux (since increased hydrogen bonding capability may increase recognition by transporters)[18] or both. Compounds **7** (large cell drop-off) and **14** (small cell drop-off) were examined in the cell assay in the presence of an efflux inhibitor.[19] Consistent with active efflux, the cell activity of **7** was increased to 0.175 μM in this system, whereas the cell activity of the already potent **14** remained unchanged at 0.010 ± 0.001 μM.[20]

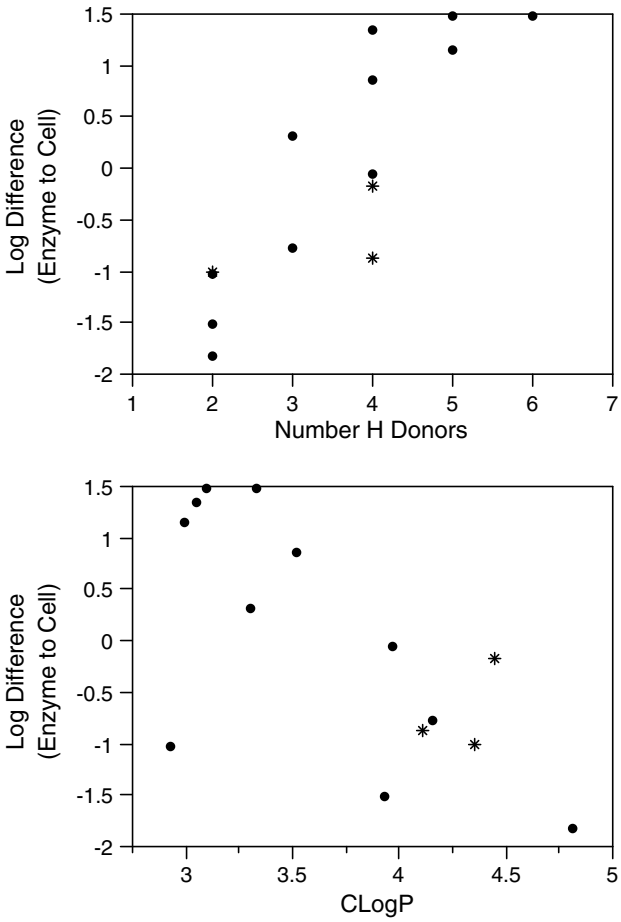


Figure 2. Plots of Log (enzyme to cell) difference versus number of hydrogen-bond donors and *Clog P* for compounds **1**–**16**. Closed circles represent 3,5-disubstituted C-2 anilines, stars represent 3-substituted anilines. Points on top line are out of range values.

In summary, crystallographic studies of a range of 3-substituted anilinopyrimidine inhibitors of EphB4 have highlighted two alternative aniline conformations are available in the active site of the kinase. In an attempt to exploit these two interactions simultaneously, a set of 3,5-disubstituted anilinopyrimidines has been prepared and these show potent enzyme inhibition. The observed range of cellular activities has been rationalised on the basis of physicochemical and structural characteristics, and has been linked, for some inhibitors, to a potential for efflux. Further studies on optimisation of both the C-2 and C-4 anilines are underway and will be reported in due course.

Acknowledgments

We thank Jonathan Wingfield for technical assistance, Eileen McCall, Isabelle Green and Anna Valentine for construct design, insect cell expression and protein preparation, and Claire Brassington for crystallisation.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.087.

References and notes

1. Kullander, K.; Klein, R. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 475–486.
2. Xia, G.; Kumar, S. R.; Masood, R.; Zhu, S.; Reddy, R.; Krasnoperov, V.; Quinn, D. I.; Henshall, S. M.; Sutherland, R. L.; Pinski, J. K.; Daneshmand, S.; Buscarini, M.; Stein, J. P.; Zhong, C.; Broek, D.; Roy-Burman, P.; Gill, P. S. *Cancer Res.* **2005**, *65*, 4623–4632.
3. Brantley-Sieders, D.; Schmidt, S.; Parker, M.; Chen, J. *Curr. Pharm. Des.* **2004**, *10*, 3431–3442.
4. Cheng, N.; Brantley, D. M.; Chen, J. *Cytokine Growth Factor Rev.* **2002**, *13*, 75–85.
5. Bardelle, C.; Cross, D.; Davenport, S.; Kettle, J. G.; Ko, E. J.; Leach, A. G.; Mortlock, A.; Read, J.; Roberts, N. J.; Robins, P.; Williams, E. J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2776–2780.
6. Human EphB4 (598–892; Y774E) was crystallised by sitting drop vapour diffusion using 2 μl 12 mg/ml protein with equivalent volumes of reservoir (25% PEG, 5000 Mme, 0.1 M Tris, pH 7.5, 0.15 M $MgCl_2$, 15% glycerol) and MPD in the drop. Crystals were robust and of space group $P2_1$ with approximate cell dimensions $a = 46.0$, $b = 53.5$, $c = 61.4$ Å and $\beta = 110.9°$. The first complex structure was solved by molecular replacement using a model of EphB2 (PDB code **1JPA**); subsequent structure solution used the newly determined EphB4 complex structures as models. Detailed protein preparation and other experimental protocols are included in the Supplementary Material to this paper.
7. Crystal structures and structure factors for EphB4 complexes with compounds **1** (resolution 2.0 Å, R-factor 0.175; compound numbering from the preceding paper),[5] **2** (resolution 1.7 Å, *R*-factor 0.238) and **7** (resolution 1.9 Å, *R*-factor 0.184) have been deposited in the PDB with accession codes **2VWU**, **2VWV** and **2VWW**, respectively.
8. EphB4 crystals were soaked in 0.5 μl compound **1** (100 mg/ml) plus 4.5 μl reservoir solution. The 1.65 Å structure was refined to $R = 16.6\%$ and deposited with PDB accession code **2VWX**. Data collection protocol and statistics are available in the Supplementary Material.
9. Complex crystal structures with compounds **2, 3, 4** and **5** were determined at 1.65 Å (except compound **4** at 2.1 Å) and refined to $R = 18.7\%$, 16.8%, 18.4% and 17.1%. PDB deposition codes are **2VWY**, **2VWZ**, **2VX0**, **2VX1**, respectively. Statistics and protocols for both parts 1 and 2 of this publication series are included in the Supplementary Material.
10. Occupancies were roughly estimated by balancing the atomic temperature factors in the two alternate ligand models and were not refined.
11. This simplistic argument ignores any impact on potency from changes in lipophilicity and/or de-solvation penalties when adding an additional substituent.
12. For detailed synthetic procedures see: Kettle, J. G.; Read, J.; Leach, A.; Barlaam, B. C.; Ducray, R.; Lambert-Van Der Brempt, C. M. P.; PCT Int. Appl. WO2007085833.
13. Belfield, A. J.; Brown, G. R.; Foubister, A. J.; Ratcliffe, P. D. *Tetrahedron* **1999**, *55*, 13285–13300.
14. Baskin, J. M.; Wang, Z. *Org. Lett.* **2002**, *4*, 4423–4425.
15. The assay detects inhibitors of recombinant EphB4-mediated phosphorylation of a polypeptide substrate in presence of magnesium-ATP using Alphascreen (Packard Bioscience) luminescence detection technology. For full details see Ref. 12.
16. 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. 8.
17. In this limited data set the correlation between number of hydrogen-bond donors and C*log P* is weak.
18. Seelig, A. *Eur. J. Biochem.* **1998**, *251*, 252–261.
19. *N*-[4-[3-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)propoxy]phenyl]-9-oxo-10H-acridine-4-carboxamide was used, an analogue of the known BCRP and PGP inhibitor Elacridar, see: Dodic, N.; Dumaitre, B.; Daugan, A.; Pianetti, P. *J. Med. Chem.* **1995**, *38*, 2418–2426.
20. This does not rule out the possibility of reduced permeability in conjunction with efflux for the less cell potent inhibitors **10** and **16**.