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Inhibitors of the tyrosine kinase EphB4. Part 3: Identification of non-benzodioxole-based kinase inhibitors

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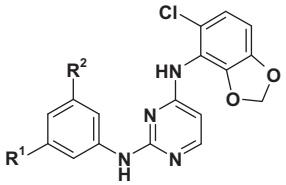
ABSTRACT

Starting from the initial bis-anilinopyrimidine **1**, good potency against EphB4 was retained when benzodioxole at C-4 was replaced by an indazole. The key interactions of the indazole with the protein were characterised by crystallographic studies. Further optimisation led to compound **20**, a potent inhibitor of the EphB4 and Src kinases with good pharmacokinetics in various preclinical species and high fraction unbound in plasma. Compound **20** may be used as a tool for evaluating the potential of EphB4 kinase inhibitors in vivo.

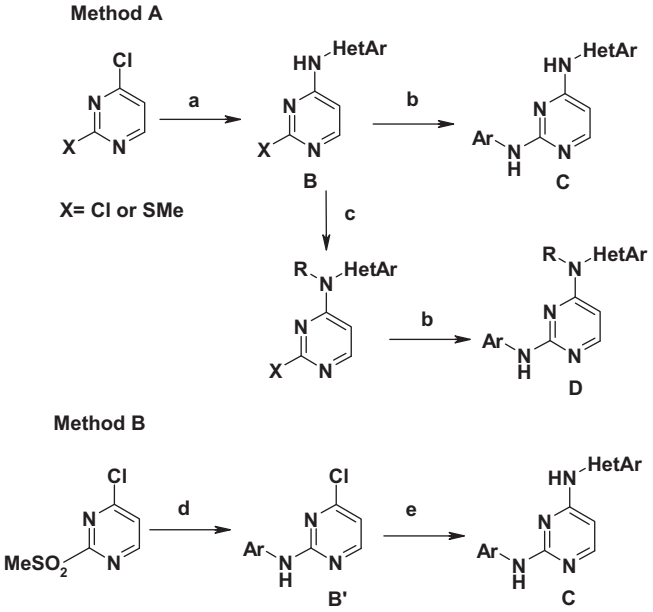
Eph receptors constitute the largest family of tyrosine kinase receptors, and together with their membrane-bound ephrin ligands, play a critical role in embryonic patterning, neuronal targeting,[1] vascular development and adult neovascularisation.[2] There is growing evidence that Eph receptor signalling may contribute to tumourigenesis in a wide variety of human cancers, either directly in tumour cells or indirectly via tumour vascularisation: for instance, Eph receptors and their ephrin ligands are frequently overexpressed in various tumour types, including breast, prostate, non-small cell lung and colon cancers.[3]

In particular, EphB4 has been one of the most studied receptor of the Eph family: inhibition of EphB4 expression using interfering-RNA or antisense oligonucleotides inhibited proliferation, survival and invasion of PC3 prostate cancer cells in vitro and in vivo.[4] Inhi-



1 R^1= SO_2Me, R^2= H
2 R^1= R^2= 4-morpholinyl

Figure 1. Compounds **1** and **2**.



Scheme 1. General synthesis of compounds **3**–**31**. Reagents and conditions: (a) for X = Cl: $HetArNH_2$, DMF or alcohols, HCl (cat.) or NEt^iPr_2, heat; or Buchwald type conditions (DBU, xantphos, Pd_2dba_3, dioxane, heat). For X = SMe: $HetArNH_2$, *n*-BuOH, HCl (cat.), 80 °C; (b) for X = Cl: $ArNH_2$, alcohols, HCl (cat.), heat; or Buchwald type conditions (DBU, xantphos, Pd_2dba_3, dioxane, heat); for X = SMe: *m*CPBA (2 equiv), DMF, 0 °C; $ArNH_2$, 2-pentanol, HCl (cat.), 150–170 °C, μw; (c) R–X (X = Br or I), K_2CO_3 or Cs_2CO_3, DMF or MeCN, rt; (d) ArNHCHO, NaH, THF, 0 °C to rt; (e) $HetArNH_2$, ^{i}PrOH, HCl (cat.), 90 °C.

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bition of EphB4 signalling using soluble extracellular-domains of EphB4 have also been shown to inhibit tumour growth in xenograft studies.[5] Transgenic studies have shown that EphB4[6] and its associated ligand ephrin B2[7] cause embryonic lethality associated with vascular modelling defects. Finally, interference with endothelial EphB4 signalling altered tumour blood vessel morphology in tumour models.[8] These observations suggest that EphB4 in particular may play a critical role in tumour angiogenesis and tumour growth.

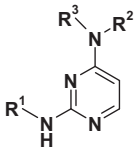
However, the functional significance of EphB4 is far from being fully understood: for instance, tumour-suppressor as well as tumour-promoting activities have been proposed for EphB4 in breast cancer.[9] In addition, by contrast with most receptor tyrosine kinases, signalling can originate from the ephrin ligands as well as from the Eph receptors; bi-directional signalling has emerged as an important mechanism by which Eph receptors and ephrin ligands control the output signal in processes of cell–cell communication. The relative contribution of these 'forward' and 'reverse' signals in tumour vascularisation and tumour growth has not been fully clarified.[10,3b]

In our search for EphB4 kinase inhibitors as potential oncology drugs, we had recently identified 4-(benzodioxolyl)amino pyrimidines such as compounds **1** and **2** as potent EphB4 kinase inhibitors (Fig. 1).[11] Previous reports have linked the benzodioxole scaffold with mechanism based P450 inhibition, which might lead to drug–drug interactions or toxicological events.[12a] This potential risk was confirmed by the following evidence: **1** and **2** showed some CYP inhibition (resp. IC_{50} 2.6 and 0.9 μM against 3A4, fluorometric assay) with evidence of time dependant inhibition.[12b] X-ray crystal structure of **1** bound to the EphB4 kinase domain[11b] has established that the benzodioxole group is buried in the selectivity pocket, the hydrophobic region beyond the Thr693 gatekeeper residue. As a consequence, variation of this group may highly impact EphB4 potency and selectivity versus other kinases.

In this publication, we describe the replacement of the benzodioxole by other bicyclic heteroaryl groups as EphB4 kinase inhibitors.

In general terms, compounds **3–31** have been made by one of the following methods,[13] as described in Scheme 1. From readily available 2,4-dichloropyrimidine (or the 4-chloro-2-methylthiopyrimidine), displacement of the more reactive 4-chloro by the heteroarylamino group under acidic, basic or Buchwald type conditions gave **B**; optional alkylation of **B** followed by displacement of the 2-chloro (or the 2-sulfonyl after oxidation of the 2-methylthio group with *m*-CPBA) under acidic or Buchwald type conditions gave the desired compound **C** or **D** (method A). Alternatively, the sequence of addition can be reversed: treatment of the 4-chloro-2-methylsulfonylpyrimidine[14] with the anion of the desired formanilide, followed by saponification afforded the corresponding 2-anilinopyrimidine **B′** with excellent regioselectivity[15]; **B′** was

Table 1
Inhibition data in an EphB4 kinase enzyme assay (using acoustic dispensing) and in a cellular assay of phospho-EphB4 inhibition for compounds **1–31**



Compound	R^1	R^2	R^3	Synthesis method	EphB4 enz. IC_{50}[a] (μM)	p-EphB4 IC_{50}[a,b] (μM)
1	3-$MeSO_2$-Ph	5-Cl-1,3-benzodioxol-4-yl	H		0.025[c]	0.187
2	3,5-Di-(morpholin-4-yl)-Ph	5-Cl-1,3-benzodioxol-4-yl	H		0.004[c]	0.014
3	3-$MeSO_2$-Ph	1,3-Benzodioxol-4-yl	H	A	0.034	0.186
4	3-$MeSO_2$-Ph	6-Cl-benzofuran-7-yl	H	B	0.300	0.897
5	3-$MeSO_2$-Ph	Benzofuran-7-yl	H	B	0.205	0.460
6	3-$MeSO_2$-Ph	2,3-Dihydrobenzofuran-7-yl	H	B	1.16	1.72
7	3-$MeSO_2$-Ph	Benzoxazol-7-yl	H	B	0.219	1.35
8	3-$MeSO_2$-Ph	Benzoxazol-4-yl	H	B	0.549	1.76
9	3-$MeSO_2$-Ph	Indazol-4-yl	H	B	0.061	2.53
10	3-$MeSO_2$-Ph	3-Cl-indol-7-yl	H	B	0.913	
11	3-$MeSO_2$-Ph	5-Cl-1,3-benzodioxol-4-yl	Me	A	0.036	0.042
12	3-$MeSO_2$-Ph	5-Cl-1,3-benzodioxol-4-yl	CH_2CN	A	0.095	0.190
13	3-$MeSO_2$-Ph	5-Cl-1,3-benzodioxol-4-yl	CH_2CH_2OMe	A	0.246	0.378
14	3-$MeSO_2$-Ph	5-Cl-1,3-benzodioxol-4-yl	CH_2CH_2OH	A	0.121	0.187
15	3,5-Di-(morpholin-4-yl)-Ph	Indazol-4-yl	H	B	0.011	0.302
16	3,5-Di-(morpholin-4-yl)-Ph	Indazol-7-yl	H	B	0.089	3.5
17	3,5-Di-(morpholin-4-yl)-Ph	Indol-4-yl	H	B	0.117	0.856
18	3,5-Di-(morpholin-4-yl)-Ph	Benzotriazol-4-yl	H	B	0.115	8.6
19	3-$MeSO_2$-Ph	Indazol-4-yl	Me	A	0.036	0.051
20	3,5-Di-(morpholin-4-yl)-Ph	Indazol-4-yl	Me	A	0.0013	0.009
21	3-(CH_2OH)-Ph	Indazol-4-yl	H	A	0.204	4.4
22	3-(CH_2OH)-Ph	Indazol-4-yl	Me	A	0.021	0.232
23	3-($CONMe_2$)-Ph	Indazol-4-yl	H	A	0.246	10.4
24	3-($CONMe_2$)-Ph	Indazol-4-yl	Me	A	0.029	0.453
25	3-($NHSO_2Me$)-Ph	Indazol-4-yl	H	A	0.084	8.42
26	3-($NHSO_2Me$)-Ph	Indazol-4-yl	Me	A	0.013	
27	3,5-Di-(morpholin-4-yl)-Ph	3-Me-indazol-4-yl	H	B	0.067	0.513
28	3,5-Di-(morpholin-4-yl)-Ph	3-Cl-indazol-4-yl	H	B	0.055	0.227
29	2,6-Di-(morpholin-4-yl)-pyridin-4-yl	Indazol-4-yl	Me	A	0.0009	0.005
30	2,6-Di-(morpholin-4-yl)-pyrimidyl-4-yl	Indazol-4-yl	Me	A	0.0026	0.024
31	4,6-Di-(morpholin-4-yl)-pyrimidyl-2-yl	Indazol-4-yl	Me	A	0.432	12

[a] Standard error is typically 0.3 log unit.
[b] EphB4 cellular autophosphorylation assay (engineered CHO-K1 cell line stably expressing EphB4).
[c] Previously reported EphB4 enzyme IC_{50} (using serial aqueous dilution)[13] for **1** and **2** was resp. 90 nM and 2 nM.[11b]

then reacted with the desired heteroarylamino derivative to give **C** (method B).

Compounds **1–31** in Table 1 were evaluated in an EphB4 kinase enzyme assay (measuring the inhibition of phosphorylation of a synthetic peptide substrate at Km ATP concentration using acoustic dispensing)[16] and in a cellular assay with a phospho-EphB4 endpoint (engineered CHO-K1 cell line stably expressing a EphB4-Myc-His construct).[13]

Replacement of the benzodioxole of **1** with other various heterocycles significantly reduced EphB4 activity (compounds **3–8**, **10**). But the indazole **9** retained good potency in the enzyme assay.

In an attempt to understand the particular role of the indazole, the crystal structure of **9** in complex with EphB4 was determined.[17] This structure, shown in Figure 2, confirms the predicted binding mode of **9** in the ATP binding site, with the pyrimidine N-1 and C-2 anilino N–H making hydrogen acceptor and donor bonds to the hinge region at Met696 and the indazole heterocycle buried in the selectivity pocket, resulting in a 'S-shaped' conformation

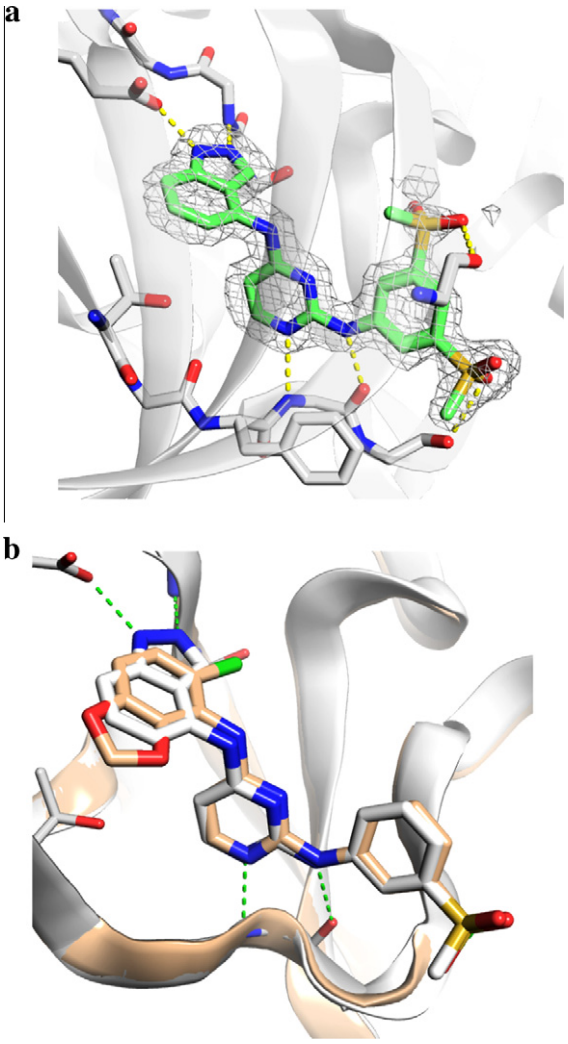


Figure 2. (a) Crystal structure (pdb:2xf9) of compound **9** bound to EphB4. Compound is shown in green. The protein backbone cartoon is represented in white. The hinge region at the bottom, E664 and D758 backbone above are represented as sticks. Refined electron density ($2f_o f_c$) contoured at 1.0 sigma represented as wire mesh. Both conformations of the C-2 aniline are shown. (b) Crystal structures of compounds **1** (white, pdb:2vwu) and **9** (wheat). Protein structures overlaid using backbone main-chain atoms. The second, minor conformation of the C-2 aniline for both compound structures is not shown for clarity. Pictures produced using PYMOL.[20]

similar to the one observed for compound **1**. However, the indazole adopts a different and flipped orientation compared to the benzodioxole **1**. Here the indazole N′1 NH and N′2 nitrogen make hydrogen donor and acceptor bonds to the side chain carbonyl of Glu664 and the backbone NH of Asp758, respectively. The 3-position of the indazole explores the same area as the 5-chloro of the benzodioxole (see Fig. 2). As observed for **1**, the C-2 aniline of **9** adopts a dual conformation within the protein structure, with the methyl sulfonyl pointing towards the hinge region or towards the Gly-rich loop.

Modification of the C-2 aniline had identified the 3,5-(dimorpholin-4-yl)aniline as a more active aniline compared to the *m*-methylsulfonylaniline (**2** vs **1**) with the chlorobenzodioxole at C-4.[11b] A similar result was observed with the 4-indazole at C-4 (**15** vs **9**). Variation of the C-4 aniline in the presence of the 3,5-(dimorpholin-4-yl)aniline at C-2 was explored. Reduced activity was observed with the 7-indazole **16**, the indole **17** and the benzotriazole **18** compared to the 4-indazole **15**, as anticipated from the key hydrogen bonds of the 4-indazole moiety with the protein. Substitution at the 3-position of the indazole with methyl or chlorine (**27** and **28**) also reduced potency slightly. Alkylation of the N-4 nitrogen on the pyrimidine was tolerated with methyl being optimal (**11–13** vs **1**) in the benzodioxole subseries. In the indazole subseries, methylation of the N-4 nitrogen showed a marked improvement of activity (**19**, **20**, **22**, **24**, **26** vs **9**, **15**, **21**, **23**, **25**).

Additive SAR was observed when combining the three key elements: N-4 methylation and 4-indazole at C-4 and 3,5-(dimorpholin-4-yl)aniline at C-2 of the pyrimidine culminated in the identification of **20** as a potent inhibitor of the EphB4 kinase. Replacement of the phenyl of 3,5-(dimorpholin-4-yl) aniline by a 4-pyridine (**29**) or a 4-pyrimidine (**30**) was tolerated, but not by a 2-pyrimidine (**31**). In the case of **31**, we hypothesised that repulsion between the lone pairs of the N-3 nitrogen of the central pyrimidine and the N-2 nitrogen of the dimorpholino-pyrimidine forces the dimorpholino-pyrimidine out of the plane of the central pyrimidine into a conformation that cannot be accommodated in the kinase.

Selectivity of **20** was evaluated against a panel of 68 kinases (Dundee panel) at 10 μM: only 12 out 68 kinases were inhibited by more than 80%, including Src-family kinases (99% and 92% of inhibition Src and Lck, respectively). At the cellular level, **20** showed good inhibition of proliferation of c-Src transfected 3T3 cells[18] (IC_{50} 2 nM) as well as autophosphorylation of EphB4 in transfected CHO-K1 cells (IC_{50} 9 nM). Some selectivity at the cellular level was observed versus other kinases involved in vascular modulation (inhibition of p-KDR in HUVEC IC_{50} 240 nM; inhibition of p-PDGFR-β in MG63 cell line IC_{50} 58 nM). It is interesting to note that scientists at GlaxoSmithKline had also identified related indazoles as inhibitors of Lck, where the indazole works as a phenol isostere.[19] The binding mode they propose based on docking studies in Lck is very similar to the binding mode we observed for **9** in EphB4.

We then looked at the pharmacokinetic properties of **20**, with the aim of using this dual Src/EphB4 kinase inhibitor as an in vivo tool. Oral administration of **20** in nude mice at 100 mg/kg gave excellent plasma levels (Table 2). Finally, **20** exhibited good pharmacokinetic parameters in rat and dog (Table 3) and relatively high fraction unbound in plasma (f_u nude mouse: 7%, f_u human: 6%)

As expected, **20** showed only modest inhibition of CYP P450 (IC_{50} 5 μM against 2C9 and 3A4, >10 μM against 1A4, 2D6 and 2C19; fluorometric assay). However, we were surprised still to see evidence of time dependent inhibition with **20**.[12b]

In conclusion, starting from the bis-anilinopyrimidine core, we have identified a potent inhibitor **20** of the EphB4 and Src kinases in a non-benzodioxole related series. This compound **20** may be

Table 2
AUC_{0-24} and plasma concentrations after p.o. administration[a] of **20** (100 mg/kg) in nude mice

AUC (μM h)	Plasma concentration (μM) at		
	1 h	4 h	24 h
48.7	11	1.9	1.8

[a] Administered as an HPMC Tween suspension.

Table 3
Pharmacokinetic parameters for compound **20**

Rat/dog Cl[a] (%hbf)	Rat/dog V_{dss}[a] (L/kg)	Rat/dog F[a] (%)
19/80	0.8/2.6	65/63

[a] Female Han Wistar rats dosed at 1.2 mg/kg iv and 5 mg/kg p.o.; mean values for male and female beagle dogs dosed at 1 mg/kg iv and 2 mg/kg p.o. Cl expressed in % of hepatic blood flow.

used as a 'relatively selective' tool for evaluating the potential of EphB4 kinase inhibitors in vivo.

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13. For detailed conditions for the synthesis of compounds **3**–**31** and description of biological assays, see: Kettle, J. G.; Read, J.; Leach, A.; Barlaam, B. C.; Ducray, R.; Lambert-Van Der Brempt, C. M. P. *PCT Int. Appl.* WO2007085833. Protecting groups for the indazolyl endocyclic nitrogen were used for compounds **19**, **20**, **22**, **24**, **26** (benzyl) or **29**–**31** (4-methoxybenzyl). For **7** and **8**, the aminobenzoxazole was introduced as the 2-Boc-NH 6-aminophenol and the 2-Boc-NH 3-aminophenol. Removal of the Boc protecting group with TFA and formation of the benzoxazole ring by treatment with trimethyl orthoformate with *p*-toluenesulfonic acid gave the expected benzoxazoles.
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17. Human EphB4 (598–892; Y774E) was crystallized as described in Ref. 11b. Detailed protein preparation, crystallization and freezing protocols are included in the Supplementary data of Ref. 11b. Diffraction data for complex of EphB4 with **9** were collected on a Rigaku FRe X-ray generator equipped with a Saturn 944 CCD detector, using a CuKα wavelength of 1.54178 Å, focused using Osmic Varimax HF mirrors at 100 K. Data were processed using MOSFLM and SCALA and reduced using CCP4 software.[20] The structures were solved by molecular replacement using coordinates of the EphB4 kinase domain[11b] as a trial model using CCP4 software. Protein and inhibitor were modelled into the electron density using, COOT[21] and AFITT.[22] The model was refined using Refmac.[23] Atomic coordinates[24] and structure factors for the EphB4 complexes with compounds **9** have been deposited in the Protein Data Bank (2xf9) together with structure factors and detailed experimental conditions.
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24. Crystallographic statistics for the EphB4/compound **9** complex are as follows: Space group $P2_1$, unit cell 47.1, 52.5, 61.4 Å, β 112.1, Resolution 56.89–1.75(1.89–1.75) Å, 26301(2619) unique reflections with an overall redundancy of 3.3(2.2) give 93.5(64.8)% completeness with R_{merge} of 6.3(35.4)% and mean I/σ(I) of 12.9(2.0). The final model containing 2108 protein, 263 solvent, and 38 compound atoms has an *R*-factor of 15.7% (R_{free} 19.4%) using 5% of the data. Mean temperature factors for the protein and the ligand are 17 and 18 $Å^2$, respectively.