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Discovery and structural analysis of Eph receptor tyrosine kinase inhibitors

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ABSTRACT

The Eph family of receptor tyrosine kinases has drawn growing attention due to their role in regulating diverse biological phenomena. However, pharmacological interrogation of Eph kinase function has been hampered by a lack of potent and selective Eph kinase inhibitors. Here we report the discovery of compounds **6** and **9** using a rationally designed kinase-directed library which potently inhibit Eph receptor tyrosine kinases, particularly EphB2 with cellular EC_{50}s of 40 nM. Crystallographic data of EphA3 and EphA7 in complex with the inhibitors show that they bind to the 'DFG-out' inactive kinase conformation and provide valuable information for structure-based design of second generation inhibitors.

The Eph/ephrin family is the largest among tyrosine kinases and is unique in that the ligands and receptors are both membrane bound providing the possibility for bidirectional cell–cell signaling. Genome analysis reveals that there are 14 Eph receptors and eight ephrin ligands. Eph receptor signaling is responsible for arguably the most diverse set of biological phenomena of any tyrosine kinase family including organ development, tissue remodeling, neuronal signaling, insulin secretion, and bone metabolism.[1,2] Not surprisingly, deregulation of ephrin-dependent signaling has been implicated in pathological conditions related to all of these systems.[3] The involvement of Eph/ephrin-signaling in tumorigenesis has been the most extensively investigated due to frequent upregulation of Eph receptor or ligand expression in numerous tumor types.[4–6] The emerging picture is complicated by the diversity of biological function that is associated with individual receptors and ligands, including oncogenic or tumor suppressor functions. The possibility of targeting Eph/ephrins therapeutically may be the most straightforward in the context of inhibiting Eph/ephrin-signaling in the vasculature as a means of preventing tumor angiogenesis.[2,6] Currently only a few small molecule Eph kinase inhibitors have been reported in the literature including 2,4-diaminophenyl pyrimidines and 2,4-diaminophenyl triazines,[7] and 3,7-diphenyl-4-amino-thieno and furo[3,2]pyridine[8] EphB4 inhibitors.

To date the majority of investigation into functions of Eph receptor tyrosine kinases has been accomplished using genetic and biochemical methods. Pharmacological approaches, which are crucial to understanding what happens upon acute inhibition of Eph kinase activity, have been hampered by a lack of potent and selective Eph kinase inhibitors. To address this deficiency we undertook a survey of a collection of known kinase inhibitors for their ability to target Eph kinase activity using an EphB2 kinase activity-dependent cellular assay. Specifically we developed a cellular assay where the murine pre-B cell line Ba/F3 was transformed with DNA encoding a fusion protein between Tel and the kinase domain of EphB2 such that the cells are capable of growing in the absence of interleukin-3.[9] EphB2 has been identified as a target of dasatinib and nilotinib by proteomic[10] and cellular screening[9] approaches. As nilotinib is known to be a considerably more selective inhibitor than dasatinib, we decided to use nilotinib as a lead compound and prepared a combinatorial library that should be able to mimic the 'DFG-out' binding mode that nilotinib utilizes to bind to Abl.[11]

Targeting of the kinase 'DFG-out' conformation is relatively common; inhibitors of this class are termed type II inhibitors.[12] An analysis of the structural features of these inhibitors reveals that they conform to a pharmacophore model that consists of a heterocyclic 'head' motif that recognizes the kinase hinge region, a linker segment that traverses across the kinase 'gatekeeper' residue and a 'tail' motif that occupies a hydrophobic pocket made accessible by the flip of the 'DFG-out' motif.[13] A solution-phase

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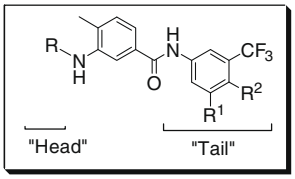
combinatorial library of several hundred compounds was synthesized where diverse heterocyclic 'head' motifs were attached to three distinct linker-tail motifs (Fig. 1). The linker-tail motifs were chosen to mimic functionality found in type II inhibitors such as nilotinib,[11] NVP-AST487,[14] and sorafenib.[15] Library synthesis consisted simply of reacting an aniline from the linker-tail motif with head groups using palladium mediated amination or amide-bond forming chemistries. The compounds were purified by mass-triggered reverse phase high pressure liquid chromatography and characterized by mass and ^{1}H NMR.

Using the Ba/F3 cellular assay, the library was screened in dose–response format against a panel of 30 distinct tyrosine kinase transformed Ba/F3 cells including Tel-EphB2.[9] This screen revealed that a subset of the library possessed inhibitory activity against EphB2, c-Kit, Abl, Bcr-Abl, PDGFR, and FGFR2/3/4 (Supplementary Table 1). Some compounds exhibited cross reactivity with Bcr-Abl (inhibitors **1**, **3**, **7**, **8**) or FGFR2/3/4 (inhibitor **10**). Abl, PDGFR, and c-kit were the most frequently inhibited kinases by this combinatorial library which is consistent with these kinases being known to be sensitive to type II inhibitors such as imatinib and nilotinib. The most potent and selective EphB2 inhibitors to emerge from this initial library were inhibitors **6** (EC_{50} = 40 nM) and **9** (EC_{50} = 40 nM). Considerably less selective EphB2 inhibitors were obtained such as **7** which also inhibits Ba/F3 cells transformed with Tel fusions of EphA3, Kit, Fms, KDR, FLT1, FGR, Src, Lyn, Bmx, and Bcr-Abl with EC_{50}s below 500 nM.

Compounds that inhibited Eph kinases (**6**–**9**) were further characterized for specificity by screening against a panel of 402 kinases that included a diverse array of serine/threonine and lipid kinases as well as tyrosine kinases using the Ambit Biosciences KinomeScan screening platform, which measures the relative binding of the target molecule to each kinase.[16] The compounds were tested at a single concentration of 10 μM (Supplementary Excel file I), and compounds that resulted in greater than ninety percent displacement relative to the DMSO control were subject to dose–response determination of a dissociation constant. Inhibitors **6**, **8** and **9** exhibited a remarkably similar selectivity profile that consisted of binding to a common set of kinases including b-raf, CSF1R, DDR1, DDR2, EphA2, EphA5, EphA8, EphB1, EphB2, EphB3, Frk, Kit, Lck, p38α, p38β, PDGFRα, PDGFRβ, and Raf1. Inhibitor **7** inhibited all these kinases and many more demonstrating how introduction of the thiophene group can have a tremendous impact on kinase selectivity. Inhibitors **6**, **8** and **9** inhibited many members of the Eph family with dissociation constants ranging from 96 nM to 520 nM; EphA6, A7 and B1 were not inhibited (Supplementary Table 2). Kinase targets that were bound with dissociation constants below 100 nM were tested in biochemical kinase activity assays using the Invitrogen SelectScreen® Kinase Profiling Service (Life Technologies Corporation, Madison, WI) to determine IC_{50} values.[17] The compounds were assayed at a starting concentration of 10 μM with threefold serial dilutions at [$K_{m,app}$] ATP with the exception of b-raf, which was conducted at 100 μM ATP. The results demonstrate that compound **9** exhibits good selectivity for Eph-family kinases with moderate potency but that there is variable agreement between K_d and biochemical IC_{50} measurements (Supplementary Table 3). These differences are not surprising as the potency of inhibition by type II inhibitors has been demonstrated to be highly dependent on the phosphorylation status of the kinase, which differs between the binding and enzymatic kinase assays.[13] These compounds were also evaluated using a temperature shift assay, which monitors the affinity-dependent stability increase of proteins,[18] to identify and rank-order ligands that interact with Eph kinases, both in their untreated and fully-auto-phosphorylated forms. We found that the auto-phosphorylation status of kinases did not significantly affect delta-*T*m values generated upon addition of ligands at 50 μM. Notably, EphA3, A5, and B3 were stabilized more significantly by ligands than EphA7 (Supplementary Table 4), results consistent with the previous assays.



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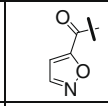
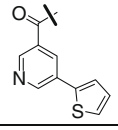
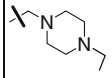
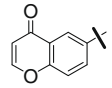
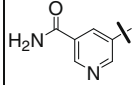
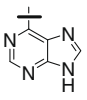
6			H
7		H	
8		H	H
9		H	H
10		H	H

Figure 1. Chemical structures of five representative library members are shown.

These results show that the Eph kinase family is also susceptible to inhibition by type II compounds and suggest that our compounds may also target the 'DFG-out' conformation. In order to investigate the structural basis for how the inhibitors recognize the Eph ATP-binding site we conducted extensive cocrystallization trials of EphA3, A5, A7, and B3 with compounds **6**–**9**. Two novel complex structures were generated: a 1.8 Å co-crystal structure of inhibitor **6** (ALW-II-38-3) with the EphA3 kinase domain (Fig. 2a, PDB code 3dzq) as well as a 2.0 Å co-crystal structures of inhibitor **9** (ALW-II-49-7) with the EphA7 kinase domain (Fig. 2b, PDB code 3dko). The apo structure of EphA7 was also determined to evaluate structural rearrangements; the apo EphA3 structure has been reported previously.[19] As expected based upon the design strategy, both type II inhibitors induce the 'DFG-out' conformation of the activation loop and preserve most of the inhibitor protein interactions that have been observed in the nilotinib-Abl co-crystal structure[11] (Supplementary movie files highlighting the rearrangement of the activation loops). Inhibitor **6** (ALW-II-38-3) forms four direct hydrogen bonds to the ATP-binding cleft of EphA3: first between the inhibitor oxazole N and the backbone NH of the hinge residue Met702, second between the oxazole amide NH and the side chain hydroxyl of the gatekeeper Thr699, third between the benzamide carbonyl oxygen and the backbone NH of Asp764 of the 'DFG-motif' and fourth between the inhibitor benzamide NH and the sidechain carboxylate of the catalytically essential Glu670 located in the αC-helix. The inhibitor binding mode mimics closely the bound conformation of nilotinib with Abl and an identical set of hydrogen bonds are formed. The 3-trifluoromethyl group is a structural feature found in many type II inhibitors such as nilotinib,[11] INNO-406,[20] sorafenib,[15] and AST487.[14] The contacts formed by this group appear to be generally accessible in most co-crystal structures of inhibitors with this structural feature suggesting that it can contribute to potency but not selectivity.

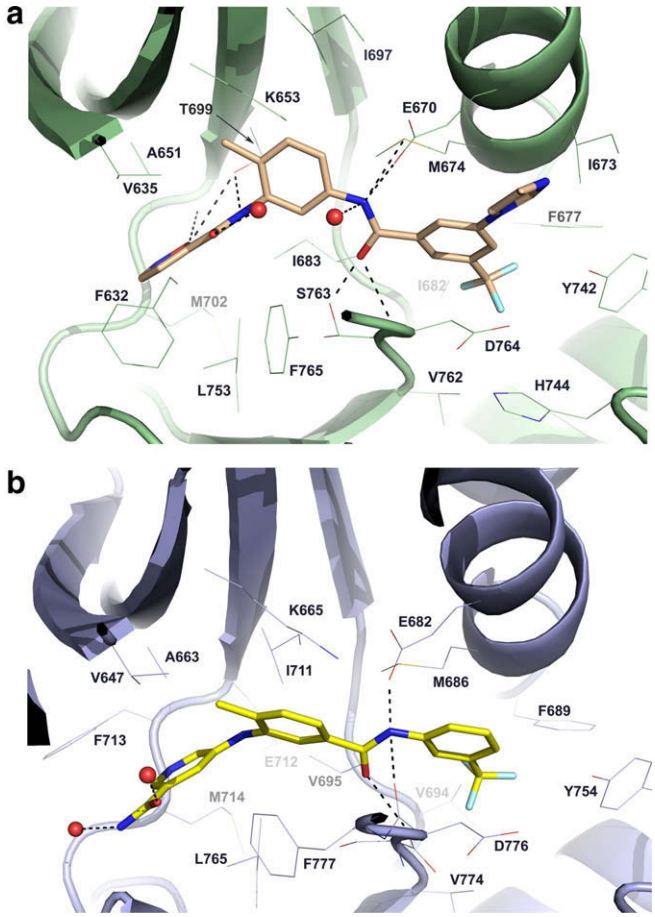


Figure 2. Complex Eph structures. (a) A slice of the active site of EphA3 in an orientation optimal for showing all direct ligand interactions. Protein is in green and compound **6** (ALW-II-38-3) in light orange with all nitrogen and oxygen atoms colored blue and red, respectively. (b) The EphA7 and **9** (ALW-II-49-7) complex structure is similarly shown, except protein is in purple and the ligand is colored yellow. Secondary structures are shown in cartoon, protein residues in line, and compounds in stick PyMol format. All potential hydrogen-bonding interactions, with distances of less than four Angstroms, between ligand and protein are shown as black, dashed lines.

Despite its relatively low affinity for EphA7, inhibitor **9** did cocrystallize with the kinase domain binding to the DFG-out conformation. As with the apo EphA3, the apo EphA7 structure is in the DFG-in conformation, where the side chain of Asp (764 in EphA3 and 776 in EphA7) in the DFG motif points into the active site, where it normally coordinates a Mg^{2+} ion. Inhibitor **9** makes three hydrogen bonds to EphA7 that mimic those between inhibitor **6** and EphA3: first between the pyridine N and the backbone NH of the hinge residue Met714 and a second between the inhibitor benzamide carbonyl oxygen and the backbone NH of Asp776 of the 'DFG-motif' and a third between the inhibitor benzamide NH and the side chain carboxylate of Glu682. Inhibitor **9** is unable to make a hydrogen bond to the gatekeeper residue because the gatekeeper residue of EphA7 is an isoleucine and not a threonine as is present in EphA3 and all other Eph kinases with the exception of EphA6 (Supplementary Fig. 1). The consequence of this substitution is loss of a critical hydrogen bond between the inhibitor aniline NH and the sidechain hydroxyl of the Ile711 and a displacement of the methylphenyl moiety from its conformation in the EphA3-crystal structure. Interestingly, an identical threonine to isoleucine substitution of the gatekeeper position is also the most recalcitrant of the Bcr-Abl mutations as it is not inhibited by nilotinib.[21] One major difference between EphA7 and Abl structure is the glycine rich loop which forms the 'roof' of the ATP-binding site. In the Abl-nilotinib co-structure the glycine loops folds down and over the opening of the ATP-binding site with the side chain of the loop's tyrosine forming stacking interactions with the inhibitor.[11] In the EphA7-inhibitor **9** co-structure the glycine rich loop, a portion of which is disordered, appears to stay above the ATP-cleft in a much more commonly observed conformation.

In order to evaluate the ability of compound **9** to inhibit full length endogenous EphB2 kinase activity, we used a glioblastoma cell line (U87) which was reported to express EphB2 and showed responsiveness to ephrinB1 ligand.[22] The cells were either left untreated or treated with increasing concentrations of compound **9** for 1 h in the presence of ephrinB1. Immuoprecipitation of EphB2 followed by western blot analysis with an anti-phospho tyrosine antibody revealed that compound **9** indeed inhibited ephrinB1-induced autophosphorylation of EphB2 with an EC_{50} between 100 nM and 1 μM (Fig. 3). This result is in good agreement with the data from the EphB2 kinase activity-dependent cellular assay.

We have discovered several type II inhibitors from a kinase-directed combinatorial library with different selectivity profiles for Eph receptor tyrosine kinases and other kinases. The most selective inhibitors **6** and **9** inhibit cellular Tel-EphB2 Ba/F3 activity with two digit nanomolar potency. These inhibitors can be considered pan-Eph kinase inhibitors although they exhibit substantially reduced affinity for EphA1, A6, A7 and B1. The reduced activity against EphA6 and A7 is presumably a result of these kinases not possessing a threonine at the 'gatekeeper position'. The structural basis for reduced affinity towards EphA1 and B1 is not clear. Selectivity profiling across a large panel of kinase binding assays revealed that these compounds also exhibited significant affinity for a subset of tyrosine kinases: CSF1R, Frk, Kit, Lck, DDR1, DDR2, PDGFRα, PDGFRβ and serine/threonine kinases: b-raf, p38α and p38β (Supplementary Fig. 2). The cellular activities against Tel-PDGFR and Tel-Kit have been validated using Ba/F3 assays. However, compounds **6** and **9** did not exhibit significant cellular activity against Src-family kinases (using Tel-Src and Tel-Lyn) nor against b-raf. Interestingly discrepancies between biochemical and cellular assays for kinase inhibitors have been previously reported.[23] Further characterization of the potential cellular inhibitory activity of compounds **6** and **9** against CSF1R, Frk, DDR1, DDR2, p38α and p38β will be required to validate whether the binding activity translates into inhibition of cellular kinase activity. Screening of structural analogs revealed that structural variations such as introduction of a thiophene group (inhibitor **7**) resulted in a dramatic decrease in kinase selectivity.

The synthesis of novel type II inhibitors, which contain hinge-interacting motifs with structures that extend into the hydrophobic pocket adjacent to the ATP-site, has the potential to deliver new inhibitors with unique kinase selectivity profiles. This provides an efficient method of discovering new kinase inhibitors that is complementary to high-throughput screening and medici-

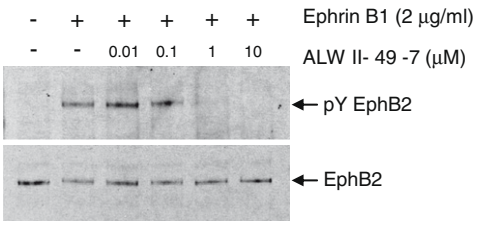


Figure 3. Compound **9** (ALW-II-49-7) inhibits EphB2 tyrosine kinase activity. The U87 glioblastoma cells were either left untreated or treated for 1 h with indicated concentrations of ALW-II-49-7 in the presence of ephrinB1. Total lysates were subject to immunoprecipitation with an anti-EphB2 antibody, and immunoprecipitated EphB2 was analyzed by western blot with an anti-phosphotyrosine antibody (top). The blot was reprobed with an anti-EphB2 antibody to show equal loading of proteins (bottom).

nal chemistry approaches. The scaffolds described here in conjunction with the crystallographic data should provide good starting points for the design of inhibitors to specific Eph receptor tyrosine kinases.

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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.2009.05.029.

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