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# Original article

# Fragment based lead discovery of small molecule inhibitors for the EPHA4 receptor tyrosine kinase

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# ABSTRACT

The *in silico* identification, optimization and crystallographic characterization of a 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinolin-1-amine scaffold as an inhibitor for the EPHA4 receptor tyrosine kinase is described. A database containing commercially available compounds was subjected to an *in silico* screening procedure which was focused on finding novel, EPHA4 hinge binding fragments. This resulted in the identification of 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinolin-1-amine derivatives as EPHA4 inhibitors. Hit exploration yielded a compound with 2  $\mu$ M (IC<sub>50</sub>) affinity for the EPHA4 receptor tyrosine kinase domain. Soaking experiments into a crystal of the EPHA4 kinase domain gave a 2.11Å X-ray structure of the EPHA4 – inhibitor complex, which confirmed the binding mode of the scaffold as proposed by the initial *in silico* work. The results underscore the strength of fragment based *in silico* screening as a tool for the discovery of novel lead compounds as small molecule kinase inhibitors.

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# 1. Introduction

The erythropoietin-producing human hepatocellular (EPH) carcinoma receptor tyrosine kinase family is the largest sub-family of receptor tyrosine kinases. These membrane spanning receptors and their ligands (ephrins) play critical roles in the developmental stage of vertebrates and are involved in processes such as axon guidance and angiogenesis [1]. There are nine EPHA receptors and five EPHB receptors in the human genome. This classification is based on early insights with respect to the activating ligands. It was believed that the EPHA receptors promiscuously interact with glycosylphosphatidylinositol-anchored ephrin-A ligands, while the EPHB receptors promiscuously interact with the transmembrane

ephrin-B ligands. More recently it has been shown that the EPHA4 receptor and the EPHB2 receptor are exceptional as they are capable of binding both A and B types of ephrins [2,3]. It has been demonstrated that the EPH receptors and ephrins are overexpressed in various tumor types [1]. For example, EPHA4, normally only expressed in the developing brain [1], was found to be highly overexpressed in tumor cells of cutaneous T-cell lymphomas (CTCL) [4], prostate cancer and pancreatic cancer [5,6]. The potential oncogenic properties of EPHA4 became evident from studies on prostate and pancreatic cancer cell lines, where downregulation of EPHA4 with siRNA reduced cell viability [7]. These results suggest that EPHA4 might be a promising target for pharmacologic intervention. Furthermore, it has been suggested that EPHA4 inhibition might be beneficial for the treatment of spinal cord injuries [8,9]. These data validate the concept of developing EPHA4 inhibitors as potential drugs and selective EPHA4 blockers are therefore desired. Although high affinity kinase inhibitors show cross activity towards

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Fig. 1. Structures of AMPPNP (1) and dasatinib (2).

EPHA4 [10,11] and small molecules can antagonize ephrin binding and EPHA4 activation [12], none of these compounds has any specificity for EPHA4. Therefore, our aim was to identify novel scaffolds capable of inhibiting the EPHA4 kinase activity. Towards this end, we designed a detailed pharmacophore screening model, which targets the well described hinge region and gatekeeper [13] residue in the ATP binding site. By using an *in silico* screening cascade, a database containing a large number of commercially available compounds was reduced to a small set of hit scaffolds, which were tested for their EPHA4 inhibitory properties.

### 2. Results

The pharmacophore screening model describes the pharmacophore features needed for interactions between the EPHA4 kinase domain (more specifically, the ATP binding site) and putative ligands. The apo structure of EPHA4 (pdb:2HEL) [14] was one of the templates used to construct the model. To accurately define ligandreceptor interacting features, the apo-EPHA4 structure was superimposed on the crystal structures of two related kinases [15]. These crystal structures contain ligands which are known to have affinity for EPHA4, namely AMPPNP and dasatinib (1 and 2, Fig. 1). AMPPNP,



**Fig. 2. A**, binding mode of AMPPNP in EPHA3 (complex **I**, pdb:2QO9). **B**, binding mode of dasatinib in ABL1 (complex **II**, pdb:2GQG). **C**, superposition of complexes **I** (green carbon atoms), **II** (orange carbon atoms) and the apo structure of EPHA4 (light pink carbon atoms, pdb:2HEL). **D**, detailed top view of superposed complexes **I** (green carbon atoms), **II** (orange carbon atoms) and apo-EPHA4 (light pink carbon atoms). **E**, annotation of pharmacophore features; F1, F5 = H-bond donor, F6 = H-bond donor projection, F3 = H-bond acceptor, F2 = H-bond acceptor projection F4 = aromatic center, F7 = H-bond donor or acceptor projection. **F**, pharmacophore screening model, constrained by the *exterior volume*. The excluded volume on the amino acid residues that make up the shape of the binding site is not shown for clarity of presentation. Annotations of pharmacophore features are similar to that in Fig. 4E.



Fig. 3. In silico screening cascade. The numbers of compounds processed in each step are indicated.

the structural analog of the endogenous substrate ATP, is expected to bind to the kinase domain in a similar fashion as ATP. Meanwhile, quantitative analysis of a variety of inhibitors using a panel of kinases revealed that the drug dasatinib has high affinity for the EPHA4 receptor [11]. The structure of EPHA3 co-crystallized with AMPPNP and the structure of ABL1 co-crystallized with dasatinib were superimposed on the apo structure of EPHA4. The binding mode of AMPPNP was derived from the EPHA3-AMPPNP complex I (pdb:2Q09, Fig. 2A) [16]. The AMPPNP adenine ring interacts with the kinase hinge region by forming two hydrogen bonds with the backbone atoms of residues Met<sup>702</sup> and Glu<sup>700</sup>. A third, water mediated, hydrogen bond to the adenine ring is formed by Thr<sup>699</sup>, the kinase gatekeeper residue. The ribose hydroxyl groups and phosphate oxygen atoms make additional hydrogen bonds to the protein, which will not be considered here.

From inspection of the ABL1-dasatinib complex **II** (pdb:2GQG, Fig. 2B) [17], it is evident that the thiazole-2-amine moiety of dasatinib makes two hydrogen bonds with the kinase hinge region by interacting with the backbone atoms of Met<sup>318</sup>. An additional hydrogen bond to the hydroxyl group of the gatekeeper residue Thr<sup>315</sup> is formed by the amide nitrogen. The orthogonally oriented 2-chloro-6-methylphenyl ring probes the ABL1 kinase hydrophobic back-pocket, a pocket that is often used in kinase inhibitor development to improve potency and increase selectivity over other kinases [13]. The apo crystal structure of EPHA4 was aligned with complexes **I** and **II**. The detailed side and top views of the superposed complexes (Fig. 2C and D) illustrate the binding modes of both ligands.

The MOE-2007 Pharmacophore Query Editor was used for the generation of the pharmacophore query based on the superposed complexes. Combining the pharmacophore features of AMPPNP and dasatinib resulted in the identification of the well known donor–acceptor–donor motif [18], visualized by the pharmacophore features F1, F3 and F5 (Fig. 2E). On the protein side, the corresponding features to F3 and F5 are shown (F2 and F6 respectively). These corresponding features in the screening model assure the correct directionality of a potential hydrogen bond donor/acceptor. The amide nitrogen atom of dasatinib forms a hydrogen bond with the gatekeeper residue Thr<sup>699</sup>, by donating a hydrogen to the hydroxyl oxygen atom of this threonine residue.



**Fig. 5. A**, GOLD-derived putative binding mode of **4** in EPHA4 (pdb:2HEL). **B**, binding mode of dasatinib in ABL1 (complex **II**, pdb:2GQG).

This is indicated by pharmacophore feature F7. The threonine gatekeeper, however, is able to interact with ligands by either accepting or donating a hydrogen atom for hydrogen bonding. Pharmacophore feature F7 is therefore annotated as donor or acceptor and F8 describes the corresponding feature to F7. Only  $\sim 20\%$  of the eukaryotic protein kinases have a threonine residue as gatekeeper [19] and specifically addressing interactions with this residue might therefore be beneficial in terms of selectivity.

The cores of the adenine and thiazole rings make up the aromatic feature F4. To constrain the size of potential hit compounds, an exterior volume filter was created (Fig. 2F) in the center of the ATP binding site. Around the carbon atom 5 of the dasatinib thiazole ring, a box of  $11 \times 10 \times 7$  (LxWxH) Angstrom was drawn in which the exterior volume was placed. The shape of the binding site was defined by the addition of an excluded volume which matched the van der Waals radii of the atoms from the residues that make up the binding site. These efforts led to a pharmacophore model that consists of eight features, which was used for the *in silico* screening of a database containing commercially available compounds.

#### 3. In silico screening cascade

The MOE-2007 Vendor Compound 3D Collection containing 2.7 million commercially available compounds, which were conformationally pre-sampled into 171.8 million conformations, was subjected to an *in silico* screening cascade (Fig. 3). A pharmacophore query using the 2.7 million commercially available screening compounds and the eight features pharmacophore model were used as input. Seven out of eight pharmacophore features had to be addressed by a molecule in order to be selected as a hit. Pharmacophore features F2, F4, F6 and F8 were marked essential. The first conformation of a compound that obeyed all criteria of the pharmacophore model was selected as a hit and the screening automatically continued with the next compound in the database. After



Fig. 4. Comparison of alternative binding poses of *in silico* hit 4 during pharmacophore placement and after docking. Projecting vectors of hydrogen bond donors (purple), hydrogen bond acceptors (magenta) and aromatic centers (orange) are shown. **A**, structures of **3** and *in silico* hit 4. **B**, placement of **4** in the pharmacophore screening model. The EPHA4 hinge and gatekeeper residues are shown for illustration. **C**, placement of **4** in EPHA4 after GOLD docking. Dashed lines indicate hydrogen bonds. The pharmacophore model is added as illustration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

#### Table 1

Inhibition of EPHA4 kinase domain by purchased compounds 5–16.



 $^{a}$  Measured by the inhibition of Poly(Glu, Tyr) phosphorylation at a compound concentration of 200  $\mu$ M.

clustering and removal of structural analogs, this screen yielded a total of 450 hits.

Next in the *in silico* screening cascade was an energy minimization step. The pharmacophore hit conformation of a compound was energy minimized inside the apo-EPHA4 binding site. During this experiment, the protein was kept completely rigid, with the exception of the gatekeeper threonine hydroxyl group. The behavior of each compound was carefully monitored during this energy minimization step. Compounds that retained hydrogen bond interactions with the protein hinge and gatekeeper residue were selected for further investigation. However compounds that lost these key interactions, or were even expelled from the protein binding site, were discarded.

The application of the energy minimization step yielded a set of 195 molecules that conformed to the criteria above. The molecules were subjected to a docking experiment using GOLD. The apo-EPHA4 crystal structure, in which we introduced one mutation, was used for these docking studies. The sidechain of Lys<sup>653</sup> was mutated into a methionine sidechain in order to overcome unwanted bias towards ionic interactions with the protonated nitrogen atom of the lysine. Ten poses for each compound were generated. Scoring and ranking of poses in the docking of fragments and drug-like molecules is still very challenging [20] and therefore the poses were visually inspected. Here, the placements of the docked compounds were compared with the energy minimized poses of the same compounds generated in step 3 of the screening cascade. Compounds that maintained key interactions (i.e., hydrogen bonding) with the site were selected for further investigation. However, compounds that were docked with binding modes which deviated from the energy minimized poses, but were involved in hydrogen bonding with the hinge and gatekeeper residues, were also selected. This resulted in the identification of 80 compounds which were selected for purchase. Based on the availability, pricing and delivery times of these scaffolds, 27 compounds (including close structural analogs of in silico hits that were unavailable) were purchased at different vendors. The inhibitory properties on the EPHA4 kinase domain of these compounds were tested in a phosphorylation inhibition assay. This assay resulted in the identification of 6,7,8,9-tetrahydro-3H-pyrazolo[3,4-c]isoquinolin1-amine (**3**, Fig. 4A), a direct analog of *in silico* hit **4** which was unavailable for purchase. Compound **3** exhibited 80% inhibition of the EPHA4 kinase activity at 500  $\mu$ M.

# 4. Hit optimization

Results from the pharmacophore screen and docking experiments were used to optimize the affinity for EPHA4. The putative binding mode of **3** was derived from the *in silico results* of the structural analog **4** (Fig. 4A). The proposed binding mode of **4**, after pharmacophore placement, is shown in Fig. 4B. The 5,6,7,8-tetrahydroisoquinoline aromatic center matches the aromatic feature F4, while the nitrogen atom matches the H-bond acceptor feature F3. The vector projecting from this nitrogen atom intersects feature F2 that was marked essential. Pharmacophore feature F5 is probed by the pyrazolo NH group and the vector projecting from this hydrogen bond donor extends towards the essential feature F6. The other pyrazolo nitrogen atom matches the acceptor/donor feature F7 and projects its hydrogen atom towards the essential feature F8. Upon docking of **4** using GOLD, however, a different



**Scheme 1.** Synthesis route for analogs of **3.** Reagents and conditions: (a) piperidine, benzene, reflux; (b) (i) RCOCI, Et<sub>3</sub>N, 1,4-dioxane, reflux (ii); HCI aq, reflux, 2 h; (c) (i) LDA,  $-78 \,^{\circ}$ C, THF (ii); RCOCI,  $-78 \,^{\circ}$ C; (d) cyanoacetamide, Et<sub>3</sub>N, EtOH, r.t. or  $\Delta$ ; (e) POCI<sub>3</sub> 160  $^{\circ}$ C, microwave; (f) N<sub>2</sub>H<sub>4</sub>•H<sub>2</sub>O 80%, reflux.



Fig. 6. Dose dependent inhibition of recombinant EPHA4 kinase activity (measured by Poly(Glu, Tyr) phosphorylation) by compounds 3 and 61–74 (n = 2).

binding mode for this scaffold was observed (Fig. 4C). In this pose, the scaffold is both rotated and flipped compared to the pharmacophore placement. Thus, the 5,6,7,8-tetrahydroisoquinoline nitrogen atom is involved in hydrogen bonding with the threonine hydroxyl group of the gatekeeper. The pyrazolo NH group forms a hydrogen bond with the carbonyl oxygen atom of Glu<sup>700</sup>, while the other pyrazolo nitrogen atom is engaged in hydrogen bonding with the Met<sup>702</sup> backbone NH group. The NH2 group of **4** forms a fourth hydrogen bond with the backbone carbonyl oxygen atom of Met<sup>702</sup>. Superimposing this binding mode with the pharmacophore features are hit, including the marked essential features F2, F6 and F8.

Inspection of Fig. 4C reveals that essential aromatic feature F4 is not contacted by **4** in the GOLD docking pose, as such, the scaffold

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Data collection	and ref	inement	statistics.

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Data statistics	EPHA4- <b>73</b>
Pdb entry	2XYU
Temperature	100 K
Space group	p2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Cell dimensions (Å) (a,b,c)	32.54, 91.54, 97.95
Resolution limits (Å)	43.19-2.12
Rmerge	0.138
No. of reflections/used	62204/54601
Completeness (%)	98
Redundancy	3.6
Ι/σ(Ι)	8.5
Refinement statistics	
Rworking (%)	18.94
Rfree (%)	22.98
r.m.s.d from ideal values	
Bond length (Å)	0.009
Bond angle (°)	1.024
Average B-factor (Å2)	20.96

would have been discarded during a pharmacophore screen which required that all eight pharmacophore features had to be addressed. However, a pose similar to the pharmacophore placement of **4** was not observed during the GOLD docking experiment. The putative GOLD-derived binding mode indicates that the methyl group of **4** is pointing towards the hydrophobic back-pocket of EPHA4 (Fig. 5A). In the ABL1 kinase complex **II**, this hydrophobic back-pocket is occupied by the 2-chloro-6-methylphenyl moiety of dasatinib (Fig. 5B).

Upon superimposition of both complexes (image not shown), the methyl group of **4** is located near carbon atom 1 of the 2-chloro-6-methylphenyl moiety of dasatinib. Thus, if the proposed binding mode of **4** is correct, growing into this hydrophobic back-pocket on the methyl side of the fragment should be allowed and result in increased affinity for EPHA4. To verify this hypothesis, initial hit exploration was performed by screening of purchased compounds **5–16** in single point measurements at 200  $\mu$ M compound concentration for inhibition of EPHA4 kinase activity. Although



**Fig. 7.** Structure of the complex of EPHA4 with **73**. The protein backbone is shown as a ribbon diagram. The compound and key residues of the protein with which it interacts are shown in a bond representation. The simulated annealing omit-map Fo-Fc electron density map contoured at 2.0  $\sigma$  of **73** is also presented in mesh format. The residues involved in hydrogen bonds are shown in bold.



Fig. 8. Heat map of % inhibition of 73 against 124 different protein kinases.

structure activity relationships cannot be determined using this data (Table 1), they are consistent with the proposed binding hypothesis. Presuming a similar binding mode for compounds **3**, **4**, **7** and **11**, the data indicate that introducing a phenyl substituent at this 5 position of scaffold **3** is allowed. We therefore synthesized a series of 5-substituted 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]iso-quinolin-1-amine analogs.

#### 5. Synthesis and activity assessment

The synthetic sequence is partially based on the work of Rosowsky and co-workers [21]. Analogs with different substituents on the 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinoline core were synthesized from cyclohexanone 17. as shown in Scheme 1. 1.3-Diketones 19-32 were synthesized via a Stork acylation reaction of intermediate **18** with the appropriate acid halide, or by deprotonation of 17 with lithium diisopropylamide and subsequent reaction with the appropriate acid halide. 1,3-Diketones 19-32 were then reacted with cyanoacetamide to yield nitriles 33-46, which upon heating in phosphorus oxychloride yielded chlorides 47–60. Treatment of 47–60 with hydrazine hydrate at reflux yielded the 5-substituted 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*] isoquinolin-1-amine products 61-74. Proof for the correct regiochemistry of the substituted 5,6,7,8-tetrahydroisoquinolines 33-46 (Scheme 1, step d) is provided by 2D-NMR analysis on either compounds **33–46**, or on final products **61–74**, and is presented in the Supporting Information.

The synthesized compounds were tested for dose dependent inhibition of EPHA4 kinase activity (Fig. 6). Growing scaffold 3 with a phenyl ring on the 5 position (71), results in an increased affinity for EPHA4. This indicates that, in line with our hypothesis, the hydrophobic back-pocket can accommodate a phenyl ring. The reduced activities of compounds 61 and 62, that both have a phenyl ring connected to the core via a carbon spacer, indicate that the phenyl ring of **71** indeed probes a pocket and that limited amount of space is available for the ring as the inhibitory effect on EPHA4 for both of these compounds is almost completely abolished. This is further illustrated by compound 63, which can be considered inactive on EPHA4 as well. The naphthalene moiety of 64 is apparently sufficiently small to fit into the pocket thereby regaining some affinity for the kinase. Reducing the size of the para substituent resulted in the recovery of the inhibitory properties of the scaffold to the level of 71 as indicated by compounds 65, 67, 68 and **70**. Switching from *para* methyl substitution to *meta* substitution (66) was allowed and did not result in a decrease in inhibition, compared to 67.

The ortho-fluorine substituted compound **69** is equally potent to **70** and further increasing the size of the ortho substituent resulted in a significant increase in potency. Compounds **72–74** completely inhibited EPHA4 activity and exhibited IC<sub>50</sub> values of 8.1  $\pm$  1.5  $\mu$ M, 4.5  $\pm$  0.5  $\mu$ M and 2.0  $\pm$  0.2  $\mu$ M respectively.

#### 6. EPHA4 – 73 complex

To confirm the predicted binding mode of these scaffolds, efforts to soak compound **73** into the available crystal of the EPHA4 kinase domain were undertaken. Crystals of apo-EPHA4 (kinase domain aa 606–846) were grown and the structure was elucidated at 1.5 Å (to be submitted). Crystals of the apo protein were soaked in 30 mM **73** to obtain the complex, flash-frozen and used to collect diffraction data to a resolution of 2.12 Å. The structure of the complex was solved using molecular replacement with the apo protein serving as the search model. The refined structure has been deposited at the protein data bank under the access code: 2XYU. An overview of the quality of the structure is presented in Table 2.

Alignment of the structures of the apo kinase domain and the complex with **73** indicates that the structure of the kinase is unchanged (r.m.s deviation of 0.179Å over 228 C $\alpha$  atoms). As predicted, compound **73** makes 4 hydrogen bond contacts within the hinge region of the kinase domain, between the tetrahydroisoquinoline core and Met<sup>702N</sup>, Met<sup>702O</sup>, Glu<sup>700O</sup> and Thr<sup>699OG1</sup> (Fig. 7). The 5-fluoro-2-methylphenyl substituent makes extensive van der Waals interactions with Lys<sup>653</sup>, Glu<sup>670</sup>, Met<sup>674</sup>, Ile<sup>683</sup> and Ile<sup>697</sup>. The  $\alpha$ C helix is in the ATP proximal position allowing the formation of the conserved Lys<sup>653</sup>-Glu<sup>670</sup> salt bridge while the activation loop is in the "DFG-in" conformation, both of which are indicative of a kinase in the active state [22,23].

# 7. Selectivity profiling

Compound **73** was screened for inhibitory activity against a panel of 124 protein kinases at 10  $\mu$ M compound concentration. Included in this panel are multiple members of the EPH kinase family, all of which were fully inhibited at this concentration (Fig. 8). In addition, **73** showed activity against a small number of other protein kinases (including BTK, Lck, and YES1). A table presenting the percentage residual kinase activity is available in the Supporting Information. Collectively, **73** seems to be a moderately selective kinase inhibitor, illustrating the use of this scaffold in targeted inhibition of EPHA4 and other members of this family.

#### 8. Conclusions

In this work we describe the fragment based discovery of a novel EPHA4 hinge binding compound. Using the structural information derived from crystal structures of two ligands bound to related kinases, a mixed pharmacophore model for the binding site of EPHA4 was constructed. *In silico* screening efforts, using a virtual library containing commercially available compounds, led to the identification of a 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*] isoquinolin-1-amine fragment, Subsequent optimization of this scaffold, by growing into the kinase hydrophobic back-pocket, resulted in the identification of an EPHA4 inhibitor with 2 µM  $(IC_{50})$  activity. An analog of this compound was successfully soaked into a crystal of the EPHA4 kinase domain, confirming the predicted binding mode of this scaffold (pdb:2XYU). Selectivity profiling against 124 protein kinases revealed that this compound seems to be a moderately selective kinase inhibitor.

The potential applicability of this scaffold was recently confirmed with the disclosure of a patent and publication by a group of researchers from Merck [24,25]. The reported  $IC_{50}$  values of the two compounds are consistent with the data presented in this work and underscores the potential of our scaffold as a possible new drug for the treatment of cancer and neuronal injuries by inhibition of the EPHA4 receptor tyrosine kinase.

#### 9. Experimental Section

#### 9.1. Synthesis. General Remarks

Chemicals and reagents were obtained from commercial suppliers and were used without further purification. Yields given are isolated yields. Flash column chromatography was carried out on a Biotage Isolera One flash chromatography system, using prepacked Biotage SNAP Cartidge KP-Sil columns with the UV detector operating at 254 nm. All <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker-250, Bruker-400 or Bruker-500 NMR. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-20AD liquid chromatograph pump system with a Shimadzu SPD-M20A UV-vis detector with the MS detection performed with a Shimadzu LCMS-2010 EV liquid chromatograph mass spectrometer. The analyses were performed using the following conditions: Xbridge (C18) 5  $\mu$ m column (100 mm  $\times$  4.6 mm) with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid), flow rate of 1.0 mL/min, start 5% B, linear gradient to 90% B in 5 min, then 5 min at 90% B, then 5 min at 5% A, total run time of 15 min. Compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at 254 nm. The purity of synthesized compounds 3 and 61-74 was confirmed to be >95%. HRMS analyses were conducted using an Agilent 1200 series pump system with the MS detection performed with a Bruker MicroTof Q mass spectrometer. Synthetic procedures for the synthesis of 18-74 are presented in the Supporting Information.

#### 9.2. In silico screening

The pharmacophore features were assigned by the MOE-2007 Pharmacophore Query Editor, after selecting the atoms of interest on both the ligands and protein. The aromatic feature was generated by manually selecting the atoms of the adenine ring of AMPPNP and the thiazole ring of dasatinib. Default radii (1Å) were used for the size of the features, except for features F4 and F8 (see Fig. 2) for which a radius of 1.2Å was used. The actual set consists of 2,824,291 non-redundant compounds (both fragments and ligands) collected from 31 vendors. MACCS Structural Keys (Bit packed) were used for calculating fingerprints of hits. Jarvis-Patrick clustering method was used as the fingerprint clustering algorithm. GOLDScore was used as scoring function for GOLD docking. Typical scores were between 12.1181 and 41.7002 and for the selected compounds between 21.2187 and 39.0420.

#### 9.3. Kinase inhibition assay

Kinase activity was measured using the ADP Hunter Plus assay kit according to the manufacturers (DiscoveRx, Birmingham, UK) recommendations. In this assay ATP turnover, and the subsequent generation of ADP, is measured in a two-step process. Reaction mixtures (20 µl) were prepared in black polypropylene 384 well flat bottom plates (Greiner, Stonehouse, UK) and contained 100 nM His-tagged EPHA4-KD, 33 µM ATP (determined as Km for this kinase preparation), 200 µg/mL E4:Y1 (Sigma, St. Louis, MO) as substrate and increasing concentrations of inhibitors. Assay buffer (pH 7.4) contains 20 mM NaCl. 15 mM Hepes. 20 mM MgCl2. 1 mM EGTA and 0.1% Tween20. After 75 min at 30 °C. reagents A and B from the kit were added and the fluorescence intensity was measured with a Tecan infinite F200 Reader (Tecan, Grödig, Austria) after 60 min incubation at 30 °C, with an Ex of 535 nm and Em of 590 nm and 25 reads per well. Standard curves, ranging from 30 nM to 75 µM ADP (dynamic range of the kit), were used as an internal assay control. Background was measured in reactions containing no substrate and subtracted from those containing E4:Y1. Dasatinib was used as a positive control. For confirmed hits, the effects of the inhibitors on the enzymes in the ADP detection step were determined in assay buffer and the reagents A and B only.

#### 9.4. Crystallography

The crystal structure of apo-EPHA4 kinase domain and the kinase domain bound to dasatinib have been elucidated. The methods and results will be reported elsewhere (manuscript in preparation). Briefly, purified apo kinase domain was used to grow crystals at 20 °C using the sitting-drop vapour-diffusion method. The purified EPHA4 kinase domain was mixed with an equal volume of a reservoir solution containing 15-21% polyethylene glycol 10 K. 100 mM ammonium acetate and 100 mM bistris pH 5.5. Rod-like crystals appeared after 1–3 days. Crystals of apo protein were soaked in drops of compound 73 at 30 mM. After 6 h incubation at 20 °C, crystals were flash-frozen using 20% glycerol plus the reservoir solution. Diffraction data was collected at 100 K at the ESRF. Datasets at 2.12 Å resolution were merged and processed using XDS [26] and Pointless [27] from the CCP4 [28] suite and the structure was solved by molecular replacement using AMoRe [29]. The apo-EPHA4 kinase domain was used as a search model. The structure was subsequently refined using PHENIX [30] and COOT [31] was used for model building.

#### Appendix. Supplementary material

Detailed synthesis procedures. Spectral and chemical characterization of compounds **33**–**74**. Table with kinase selectivity profiling results. Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech. 2011.11.020.

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