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Discovery of a Novel Chemotype of Tyrosine Kinase Inhibitors by Fragment-Based Docking and Molecular Dynamics

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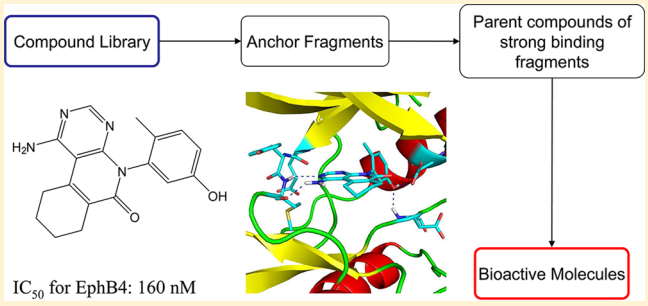
Supporting Information

ABSTRACT: We have discovered a novel chemical class of inhibitors of the EphB4 tyrosine kinase by fragment-based high-throughput docking followed by explicit solvent molecular dynamics simulations for assessment of the binding mode. The synthesis of a single derivative (compound 7) of the hit identified in silico has resulted in an improvement of the inhibitory potency in an enzymatic assay from 8.4 μM to 160 nM and a ligand efficiency of 0.39 kcal/mol per non-hydrogen atom. Such remarkable improvement in affinity is due to an additional hydroxyl group involved in two favorable (buried) hydrogen bonds as predicted by molecular dynamics and validated by the crystal structure of the complex with EphA3 solved at 1.7 Å resolution.



KEYWORDS: *In silico screening, EphB4 kinase, angiogenesis, cancer, explicit solvent MD*

The Eph-ephrin system, including the EphA2 and EphB4 receptors, plays a critical role in tumor and vascular functions during carcinogenesis.[1,2] Recently, it has been shown that delivery of chemotherapeutic drugs by an EphA2 targeting peptide into EphA2-expressing cancer cells led to dramatically improved efficacy in inhibiting tumor growth.[3] So far, a few Eph inhibitors have been identified, including the marketed drug Dasatinib (Figure S1 in the Supporting Information).[4−12] Although their role is still controversial for certain types of cancer, e.g., non small cell lung cancer,[13] the identification of selective inhibitors of Eph tyrosine kinases will help to elucidate their involvement in deregulated signaling.

Previously, we have developed an efficient in silico procedure called ALTA, which stays for anchor-based library tailoring approach, to interrogate a library of compounds for high-throughput docking.[14] First, small and mainly rigid virtual fragments are docked in the binding site. The fragments with most favorable calculated binding free energy (anchors) are used to identify the compounds with 2D structure containing one of these anchors, which are then submitted to flexible-ligand docking. In this letter, we report a new approach for in silico screening based on the synergistic combination of the ALTA procedure for docking followed by explicit solvent molecular dynamics simulations for further validation of the binding poses.

The flowchart of the ALTA procedure is shown in Figure 1. First, the nearly 9 million compounds in the ZINC-all now library[15] (version of August 2011) were decomposed into 563,774 fragments by in house developed software (Figure S2 in the Supporting Information). Just like its in vitro counterpart of fragment-based drug discovery,[16,17] the success of the ALTA

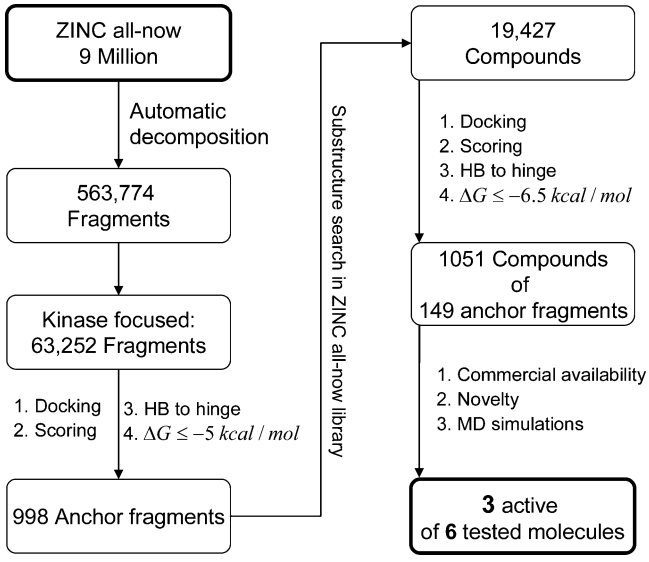


Figure 1. Flowchart of the ALTA virtual screening approach for the tyrosine kinase EphB4.

in silico screening approach depends on the choice of fragments. The use of virtual fragments by computational decomposition of a real compound library offers opportunities to explore a much greater fragmental space, with no limitations in availability. To obtain fragments with high chemical richness

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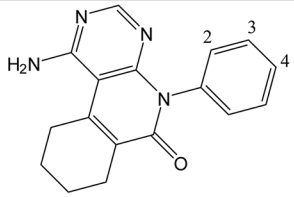
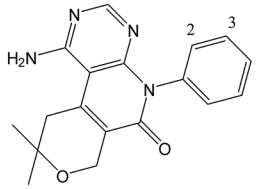
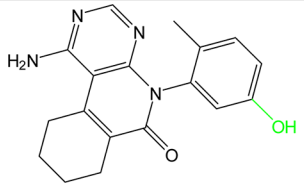
Table 1. Novel EphB4 Kinase Inhibitors Discovered by the ALTA Virtual Screening Approach with Hit Optimization Colored in Green

Compounds		Substituents	IC_{50} (μM)[a]	IC_{50} (μM)[b]	LE[c]
	**1**	2-methyl	48%[d]	8.4	0.30
	2	3-methoxy	9.4 (75%[d])	5.2	0.30
	3	4-methoxy	37%[d]	N.A.[e]	N.A.
	4	2-methoxy	5%[d]	N.A.	N.A.
	**5**	3-methoxy	38%[d]	14.3	0.25
	6	2-methoxy	0%[d]	N.A.	N.A.
	**7**		0.30	0.16	0.39

[a]Enzymatic assay based on FRET carried out in house using Invitrogen EphB4 assay kit at an ATP concentration of 30 μM. [b]Enzymatic assay with radioactive ATP at 1 μM concentration carried out at Reaction Biology Corp. The IC_{50} values were determined in a 10-dose response. [c]Ligand efficiency in kcal/mol per heavy atom. [d]Percentage inhibition at compound concentration of 30 μM and ATP concentration of 30 μM. [e]N.A.: not available.

that can serve as a starting point either directly for hit optimization or for identification of their "parent" compounds, we developed a new decomposition protocol whose main difference from our previous approach[18] is the preservation of longer substituents (e.g., *N*-methylurea) on ring systems (details of algorithm explained in Figure S2 in the Supporting Information). Most of the fragments obtained by the new decomposition algorithm have a molecular weight ranging between 150 and 300 g/mol, possess fewer than five rotatable bonds, and do not have any formal charge (Figure S3 in the Supporting Information). Second, this set of fragments was reduced to a kinase-focused library of 63,252 fragments by retaining only those with molecular weight smaller than 300 g/mol, a maximum of three rotatable bonds, more than one ring, and the capability to form two hydrogen bonds with the backbone polar groups of the so-called hinge region. For the latter criterion acidic CH groups (e.g., in aromatic rings) were also considered as donors. The requirement of having more than one ring helps to direct the search toward chemical space less affected by the crowded intellectual property coverage, given the diversity in fused rings. Moreover, one-ring anchor fragments are too small for providing enough binding energy. Use of a target-focused fragment library is computationally more efficient than docking the entire library of fragments.

The kinase-focused fragment library was then docked into the ATP binding site of EphB4 (PDB code 2VWX) by AutoDock4,[19] followed by ranking according to a previously reported scoring function.[8] The 998 fragments with an estimated binding energy lower than −5 kcal/mol were used to identify their "parent" compounds in the ZINC-all now library which yielded a total of 19,427 compounds. Flexible-ligand docking of these compounds followed by scoring[8] and elimination of those mentioned in patents related to kinases resulted in four scaffolds, which were further investigated by explicit solvent molecular dynamics simulations using the all-atom CHARMM PARAM22 force field[20] and the TIP3P model of water.[21] Two molecules sharing the pyrimidoisoquinolinone scaffold showed stable intermolecular hydrogen bonds with the hinge region (bottom panels of Figure S4 in the Supporting Information). Thus, commercially available compounds **1**−**6**, bearing a pyrimidoisoquinolinone scaffold, were purchased and tested (Table 1). Of these six compounds, **1**, **2**, and **5** showed inhibition of the EphB4 tyrosine kinase in the low micromolar range in two different enzymatic assays based on FRET (carried out in house) and radioactive ATP (performed at Reaction Biology Corp.), respectively.

The previously reported optimization of another chemical class of EphB4 inhibitors discovered by the ALTA approach[7,14] suggested to us that the addition of a hydroxyl group in position 5 of the phenyl ring in compound **1** would increase the binding affinity. This hydroxyl group is expected to be involved in two additional hydrogen bonds with the side chain of Glu664 and the backbone NH of the Asp in the DFG motif, as observed in so-called type I1/2 inhibitors[22] of tyrosine kinase with small gatekeeper residue (e.g., Thr) and DFG-in conformation. As predicted, compound **7** shows four stable hydrogen bonds with the ATP binding site (two with the hinge region and two involving the additional hydroxyl group) in two 300-ns molecular dynamics simulations with explicit solvent (Figure 2). Note that these molecular dynamics runs were started from the binding mode obtained by docking and were carried out before chemical synthesis of compound **7**.

The synthesis of compound **7** started with the saponification of ethyl 2-oxocyclohexanecarboxylate **8** in the presence of sodium hydroxide. Reaction of the β-keto acid **9** with acetone in the presence of acetic anhydride and concentrated sulfuric

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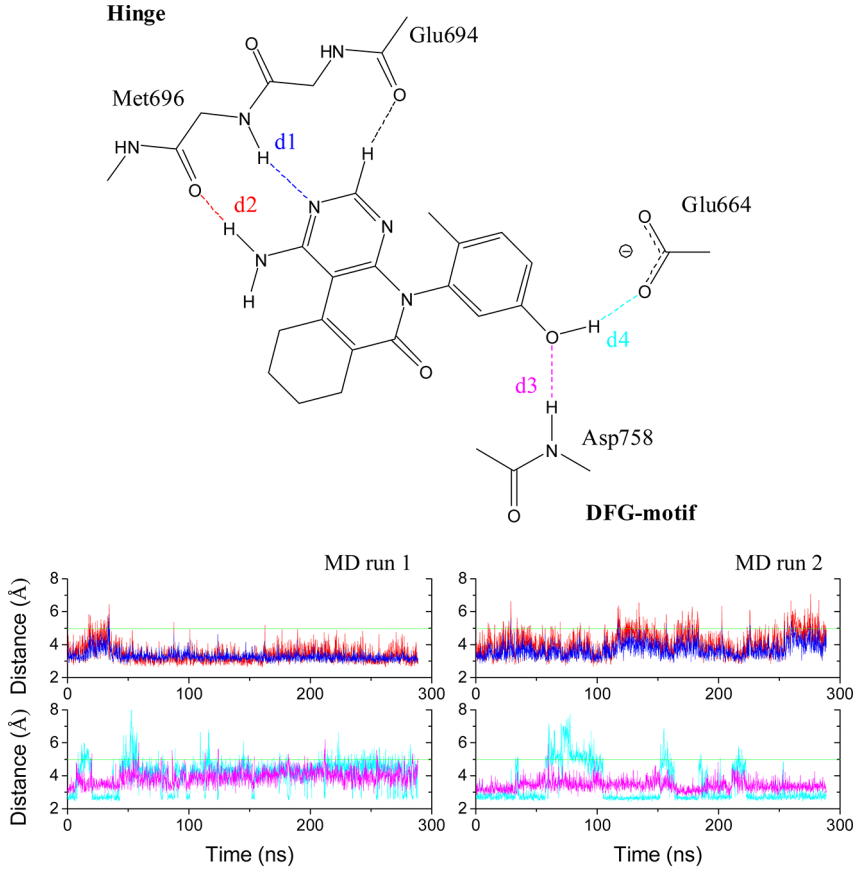
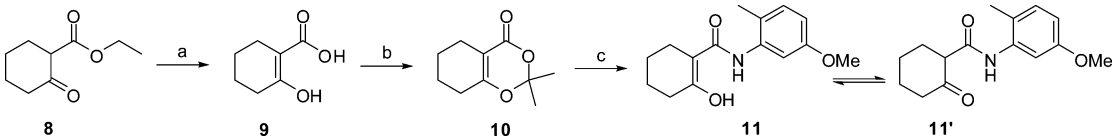


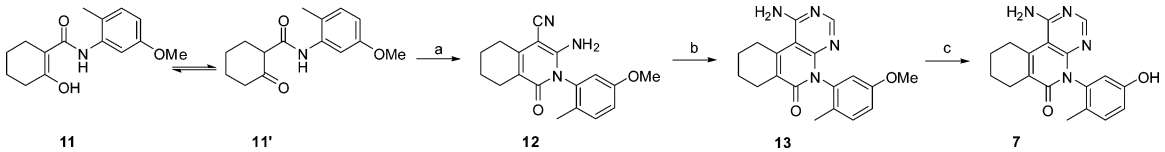
Figure 2. 2D illustration of binding mode of inhibitor **7** in the ATP binding site of EphB4 (top) and time series of intermolecular hydrogen bonds, i.e., distances between donor and acceptor atoms, in two molecular dynamics simulations started with different random values of the initial atomic velocities (bottom). The choice of colors is consistent in top and bottom panels while the green horizontal lines in the latter are drawn to illustrate that the hydrogen bonds are always formed except for transient ruptures.

Scheme 1. Synthesis of Tautomers 11 and 11′[a]



[a]Reagents and conditions: (a) NaOH, water, 25 °C, 12 h, 61%; (b) acetone, H_2SO_4, acetic anhydride, 0 °C, 4 h, 93%; (c) 5-methoxy-2-methylaniline, *o*-xylene, MW, 150 °C, 5 min, 46%.

Scheme 2. Synthesis of Target Compound 7[a]



[a]Reagents and conditions: (a) malononitrile, piperidine, EtOH, 100 °C, 2 h, 82%; (b) formamide, 210 °C, 7 h, 63%; (c) BBr_3, CH_2Cl_2, 25 °C, 16 h, 28%.

acid provided the activated acetal **10**,[23] which reacted with 5-methoxy-2-methylaniline under microwave irradiation to afford a tautomeric mixture of amides **11** and **11′** (Scheme 1).[24] Reaction of the mixture with malononitrile provided intermediate **12**, which upon condensation with formamide afforded compound **13**.[25] Final demethylation for the methoxy group in the aromatic ring was obtained in the presence of BBr_3 (Scheme 2).

It was gratifying to observe that in the enzymatic assay the potency of compound **7** is about 50 times higher than that of the parent compound **1** (Table 1). This improvement corresponds to a difference in binding free energy of about 2.5 kcal/mol, which is consistent with the two additional hydrogen bonds of the hydroxyl group of **7** as predicted by the docking and molecular dynamics simulations. The final validation was obtained by the determination of the crystal

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structure of the complex between the catalytic domain of EphA3 and inhibitor 7 (resolution of 1.7 Å, see Supporting Information for details of X-ray crystallography). The binding mode in the crystal structure confirmed the predicted binding pose of inhibitor 7 into EphB4 (DFG-in conformation) and the four intermolecular hydrogen bonds observed in the molecular dynamics runs (Figure 3). The EphA3 tyrosine kinase was used for determination of the crystal structure because of its straightforward expression in *Escherichia coli*[10] while expression of EphB4 has been reported only in insect cells.[5] Note that 32 of the 36 residues in the ATP binding site of EphA3 are identical to those of EphB4.[26] Moreover, the side chains involved in binding compound 7 are identical in EphA3 and EphB4.

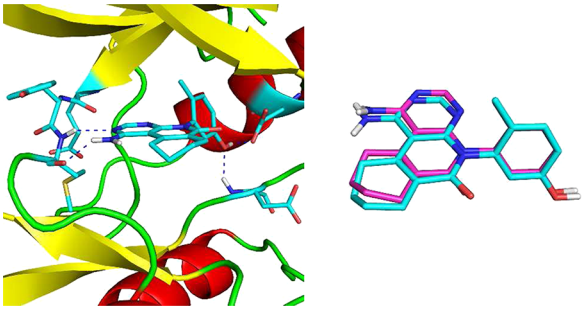

Figure 3. (Left) Crystal structure of inhibitor 7 bound to the kinase domain of EphA3. Dashed lines represent intermolecular hydrogen bonds. (Right) Superposition of pose of 7 in EphB4 obtained by docking and molecular dynamics (carbon atoms in magenta) onto the pose of 7 in EphA3 obtained by X-ray crystallography (carbon atoms in cyan). The overlap was obtained by structural alignment of the crystal structure of the EphA3/inhibitor 7 complex to the in silico predicted complex of 7 and EphB4 using only the C_α atoms of the two kinase domains.

The affinity of compound 7 was further tested on a panel of five tyrosine kinases, known as validated drug targets in cancer therapy. The IC_{50} value in enzymatic assay in the presence of the recombinant catalytic domain and 1 μM radiolabeled ATP is 0.338 μM for Src, 0.864 μM for Abl1, 1.38 μM for Lck, 1.62 μM for EGFR, while no inhibition was observed for IGF1R. Thus compound 7 has higher affinity for EphB4 than for these five tyrosine kinases.

Compound 7 was also tested in a cell-based assay. The cellular EphB4 phosphorylation assay (performed at ProQinase GmbH) makes use of the murine embryonal fibroblast cell line (MEF), which expresses a high level of exogenously introduced full-length human EphB4. Stimulation of these cells with 2 μg/mL soluble human Ephrin-B2/Fc chimera for 2 h results in receptor tyrosine autophosphorylation. Compound 7 shows a cellular IC_{50} of 0.93 μM, indicating its potential for future optimization.

In conclusion, we have discovered a new chemical class of kinase inhibitors by fragment-based docking into the ATP-binding site of the EphB4 kinase and molecular dynamics simulations for further assessment of the binding mode. The chemical synthesis of a single derivative (compound 7) by addition of just one hydroxyl group, designed on the basis of the simulation results, has resulted in a novel chemotype of nanomolar inhibitors of the EphB4 tyrosine kinase. Finally, the determination of the crystal structure of the complex of EphA3 with compound 7 has definitively validated the binding mode predicted in silico.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Chemical synthesis, crystallization, similarity comparison with known Eph inhibitors, description of the algorithm used to decompose a compound library, distribution of physicochemical properties of the library of anchor fragments, and MD screening of candidate compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The atomic coordinates and structure factors of EphA3 in complex with compound 7 have been deposited with the Protein Data Bank as entry 4G2F.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EphB4, erythropoietin producing human hepatocellular carcinoma receptor B4; ALTA, anchor-based library tailoring approach; MD, molecular dynamics

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