Bioorganic & Medicinal Chemistry Letters 24 (2014) 1523–1527

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Discovery of dual ZAP70 and Syk kinases inhibitors by docking into a rare C-helix-out conformation of Syk

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ARTICLE INFO

Article history:
Received 17 December 2013
Revised 28 January 2014
Accepted 31 January 2014
Available online 10 February 2014

Keywords:
Syk kinase
ZAP70 kinase
C-helix-out
High-throughput docking
Scoring with solvation

ABSTRACT

The non-receptor tyrosine kinase Syk (spleen tyrosine kinase) is a pharmaceutical relevant target because its over-activation is observed in several autoimmune diseases, allergy, and asthma. Here we report the identification of two novel inhibitors of Syk by high-throughput docking into a rare C-helix-out conformation published recently. Interestingly, both compounds are slightly more active on ZAP70 (Zeta-chain-associated protein kinase 70), which is the kinase closest to Syk in the phylogenetic tree of human kinases. Taken together, the docking pose and experimental results suggest that the higher affinity of the inhibitors for ZAP70 than Syk originates from a more populated C-helix-out conformation in ZAP70. The latter observation is congruent with the 100-fold lower intrinsic activity of ZAP70 than Syk, as the C-helix-out conformation is inactive. The pharmacophore features of DFG-in, C-helix-out compounds are analyzed in relation to DFG-out inhibitors.

Spleen tyrosine kinase (Syk) is a member of the cytoplasmic protein tyrosine kinase family. The validity of Syk kinase as a target for therapeutic intervention by small molecules has progressed over the past decade, and Syk has recently entered the mainstream of potential pharmaceutical targets with early indication of clinical efficacy in diverse autoimmune and inflammatory diseases.[1] The most advanced selective inhibitor of Syk, fostamatinib, is currently in clinical trials as an oral treatment of immune thrombocytopenic purpura (ITP).

The majority of small molecule inhibitors of protein kinases occupy the ATP binding site and form one to three hydrogen bonds with the hinge region that connects the two lobes of the kinase catalytic domain. The ATP binding site is highly conserved and the chemical space of these inhibitors (called type I) has become overcrowded over the years so that the design of selective small molecule inhibitors bearing novel scaffolds is challenging, thus calling for exploration of novel binding sites.

Ever since the approval of the first small molecule kinase inhibitor (Gleevec),[2] much attention was drawn to the so-called type II inhibitors.[3] Gleevec binds to the inactive state of the Abl1 kinase domain, which is characterized by a closed conformation of the activation loop. The displacement of the Phe side chain of the DFG motif (DFG-out conformation), a conserved triad at the beginning of the activation loop, induces remarkable changes in the ATP binding site and exposes an additional hydrophobic pocket. Interestingly, upon binding of the archetypal DFG-out inhibitor Gleevec, Syk maintains a DFG-in conformation.[4] An analysis of kinases for their propensity to adopt DFG-out conformations suggested that Syk is unlikely to do so.[5] However, very recently two DFG-out Syk structures were reported for the first time.[5] As observed previously for several type II inhibitors,[3] the moiety occupying the hydrophobic pocket of the DFG-out conformation is (trifluoromethyl)benzene. Although the DFG-out pocket is less conserved in sequence among kinases than the ATP-binding pocket, its hydrophobic nature is common to all kinases. It is likely that selectivity of type II inhibitors mainly originates from the differential capability of kinases to populate the DFG-out conformation, instead of sequence variation in the hydrophobic pocket. Along with the two DFG-out conformations, the report of a rare DFG-in, C-helix-out conformation of Syk is especially striking.[5] The significant movement of the C-helix results in a hydrophobic pocket comparable in size to classic DFG-out pockets. Only three c-Met ligands in the Protein Data Bank (PDB) may potentially occupy this space, in sharp contrast to 112 PDB ligands representing 16 kinases that occupy the DFG-out pocket.[5] Thus, this rare DFG-in, C-helix-out conformation may open new avenues for the design of novel selective Syk inhibitors.

Here, we report an in silico screening campaign that makes use of the C-helix-out conformation of Syk with the aim to discover novel inhibitors as a way to further validate our structure-based high-throughput docking approach.[6–9] The ZINC-all now library

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http://dx.doi.org/10.1016/j.bmcl.2014.01.083
0960-894X/

(version of 2011) was first filtered to generate a focused library with the following rules based on the features of the binding site: (1) less than 7 rotatable bonds; (2) at least one hydrogen bond donor and two acceptors; (3) number of rings from 4 to 6. Using these filters, the initial library of nearly 9 million compounds was reduced to a focused library of 873,702 molecules (Fig. 1). The focused library was then docked into the DFG-in, C-helix-out structure of Syk (PDB code 3TUC) by an in-house developed fast docking tool, which is based on a combination of simulated annealing and genetic algorithm optimization of position, orientation, and rotatable bonds of the ligand. Previously, the docking tool has led to the discovery of novel ZAP70 inhibitors.[9] The docking score mainly consists of van der Waals interaction energy and a linear distance-dependent hydrogen bonding energy with empirical weights for each donor and acceptor type. The 4,513,006 docking poses of the 282,385 compounds with docking score <−8 kcal/mol were further minimized in the rigid protein by CHARMM[10,11] with the CHARMm22 force field.[12] The poses were then subject to three filters: van der Waals efficiency (i.e., intermolecular van der Waals energy divided by molecular weight) more favorable than −0.1 kcal/g, hydrogen bonding penalty[6] smaller than 2, and presence of at least one hydrogen bond to the hinge and at least one to the DFG-motif. With these filters, unrealistic poses were removed efficiently before rescoring by a computationally expensive energy function. The 64,101 poses of the 37,517 compounds that survived these filters were ranked according to a previously reported binding energy function which is transferable among kinases,[6] and has been used to identify novel classes of kinase inhibitors with activity ranging from low micromolar to nanomolar.[6–9] Compared with the docking score, the binding energy function used for the final ranking takes into account the electrostatic solvation energy,[13] which is calculated by the finite-difference Poisson method in CHARMM,[11,13] and an efficient evaluation of the penalty for buried polar groups not involved in hydrogen bonds.[6] The 689 compounds with predicted binding energy more favorable than −9 kcal/mol were then inspected visually.

Among these 689 compounds, the single-digit nanomolar c-Met inhibitors **2**[14] and **3** ranked 229 and 27, respectively (Fig. 2). Superposition of the docking pose of compounds **2** and **3** to the crystal structure of the 100-nM inhibitor **1** in complex with Syk (PDB code 3TUC)[5] shows overlaid pharmacophore features, with minor difference in the orientation of the phenyl ring far away from the hinge region. Compared with compounds **2** and **3**, the Syk inhibitor **1** has an additional methylene linker, which allows its phenyl ring to better fill the deep C-helix-out pocket. (It has also to be noted that the three c-Met inhibitors capable of penetrating the C-helix-out pocket all have an additional linker group for the phenyl ring, i.e., –CH2– in 3EFJ and 2RFN, and –NH– in 3EFK.[5]) Since compound **2** binds to c-Met in its DFG-out conformation (3CE3),[14] the two DFG-out structures of Syk (PDB code 3TUB and 3TUD) were superimposed on 3CE3 by alignment of C_α atoms to evaluate if compound **2** could bind to Syk in its DFG-out conformation. The superposition shows some clashes between compound **2** and Syk, which are also reflected in the docking score of −7.9 kcal/mol (3TUB) and −8.9 kcal/mol (3TUD), compared with a docking score of −10.2 kcal/mol in the C-helix-out structure of Syk (3TUC).

Following the analysis of known inhibitors, 22 compounds out of the 689 top ranking ones (vide supra) were selected according to novelty and diversity (Fig. 1) for in vitro validation by a radiometric assay. Compounds **4** and **5** inhibit Syk with an IC_{50} of 22.6 and 18.4 μM, respectively (Table 1). The two compounds were further tested on a panel of four related kinases. Both compounds show no inhibition against c-Met, and 30–40% inhibition against FAK/PTK2 at the highest concentration tested (50 μM, Fig. S1), while they show single-digit μM IC_{50} values against both ZAP70

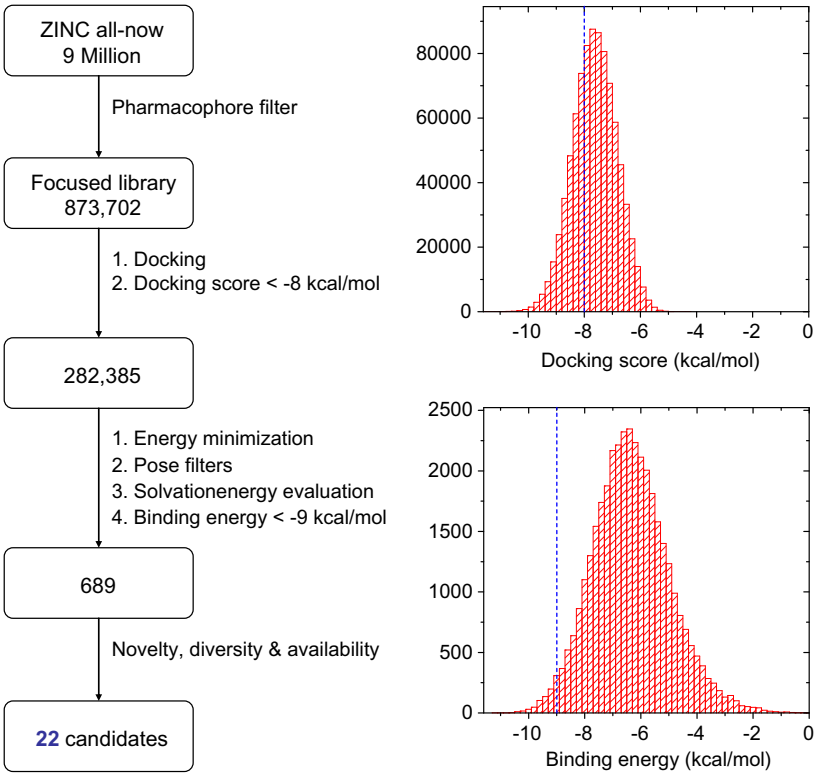

Figure 1. (Left) Flow chart of virtual screening by high-throughput docking into the C-helix-out structure of Syk (PDB code 3TUC). (Right) distribution of docking scores for the 873,702 compounds of the focused library (top), and distribution of predicted binding energies for the 37,517 compounds that passed the following three filters (bottom): (1) van der Waals energy/molecular weight ⩽−0.1 kcal/g; (2) hydrogen bonds to hinge and DFG-motif; (3) hydrogen bonding penalty ⩽2.

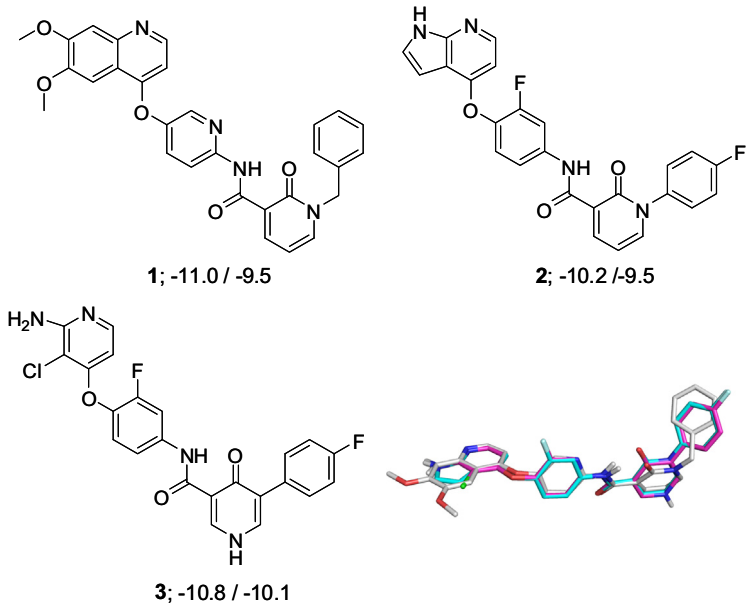

Figure 2. (Top and bottom, left) Two single-digit nM c-Met inhibitors (**2** and **3**) identified in the ZINC all-now library as potential new Syk inhibitors by the virtual screening approach described in Figure 1. The values next to the compound number are Syk docking score and predicted binding energy with electrostatic solvation both in kcal/mol. Compound **1** was reported to inhibit Syk with an IC_{50} of 100 nM.[5] Note that although inhibitor **1** is not in the ZINC all-now library it was docked and its binding energy with solvation was calculated as a basis of comparison. (Bottom, right) Superposition of the predicted binding modes of compounds **2** (carbon in cyan) and **3** (magenta) to the crystal structure of inhibitor **1** (gray) in complex with Syk (PDB code 3TUC) indicates similar interaction mode. Note that the predicted binding mode of compound **2** in Syk (DFG-in, C-helix-out) is different from its binding mode observed in the X-ray crystal structure of the complex with c-Met (DFG-out, PDB code 3CE3).

Table 1
IC_{50} values (in μM) of two compounds identified by high-throughput docking into Syk (PDB code 3TUC)

Compound	Syk	ZAP70	JAK2	FAK/PTK2	c-MET
4	22.6	8.6	8.9	>50	>50
5	18.4	4.0	3.7	>50	>50

The radiometric assay was carried out at Reaction Biology Corp. with radioactive ATP at 1 μM concentration. Compound **4** was tested as racemic mixture. The IC_{50} (i.e., the inhibitor concentration at which the enzymatic activity is reduced by 50% with respect to DMSO control) was evaluated from measurements at 10 doses with 2-fold serial dilution starting at 50 μM. Dose-response curves for compounds **4** and **5** are shown in Figure S1.

and JAK2. Compounds **4** and **5** share a pyrimidin-4(3H)-one moiety forming two hydrogen bonds with the hinge region, and further form a hydrogen bond with the backbone NH group of the Asp residue of the DFG-motif (Fig. 3). Given their single-digit μM IC_{50} against ZAP70 and scaffold novelty (SciFinder; https://scifinder.cas.org), compounds **4** and **5** could serve as a good starting point for hit optimization. ZAP70 is closely related to Syk in both homology and function, and known as an attractive therapeutic target for various allergic and autoimmune disorders as well as immunosuppressive therapy following organ transplantation.[15–19] However, a cell-permeable and highly specific ZAP70 inhibitor has not been reported as of today.[1]

The crystal structure of ZAP70 kinase in complex with an ATP analogue (2OZO) shows that the C-helix is kept away in an inactive position, a rarely observed displacement in kinases.[20] However, docking of compounds **4** and **5** into this crystal structure of ZAP70 is not possible due to the collapse of the C-helix-out pocket. To provide more evidence for the C-helix-out binding mode we investigated other binding modes. Although docking of compound **5** into the ATP-site of active ZAP70 (1U59) shows a decent binding mode (Fig. S2), the analogous of compound **5** devoid of the two fluorine atoms (which are difficult to treat by conventional force fields) unbinds within 150 ns in four molecular dynamics simulations (Fig. S3). Furthermore, although they could be docked into the ATP-site of ZAP70, the 16 derivatives of *N*-(5-benzylthiazol-2-yl)acetamide identified by substructure search (Fig. S4) do not inhibit Syk or ZAP70 at a test concentration of 50 μM, except for compound **15** which shows a moderate inhibition of 81% against ZAP70 and 37% against Syk. Taken together, these in silico and in vitro data suggest that compound **5** does not bind to the active conformation of ZAP70. Moreover, a docking study indicates that compound **5** cannot fit into any of the two DFG-out structures of Syk.

Similar to selectivity of type II inhibitors, we postulate that selectivity of C-helix-out inhibitors is determined by the capability of kinases to populate the C-helix-out conformation. It has been reported that the intrinsic activity of Syk is up to 100-fold higher than the one of ZAP70.[21] Moreover, 10-μs long molecular dynamics simulations of Syk and ZAP70 with an enhanced sampling technique[22] suggest that ZAP70 has a higher propensity than Syk to reside in a C-helix-out conformation (data not shown). On the other hand, we cannot completely rule out that the higher affinity of compound **5** for ZAP70 thank Syk is due, at least in part, to the few different residues in the ATP binding site and/or adjacent pockets.

Finally, it is interesting to compare C-helix-out inhibitors with DFG-out inhibitors in terms of pharmacophore features from a structural perspective (Fig. 4). Similar to DFG-out binding site, the C-helix-out binding site also consists of three sub-pockets: (1) the hinge region; (2) the newly exposed C-helix-out pocket; and (3) a linker region connecting the hinge region and the C-helix-out pocket. The majority of DFG-out inhibitors utilize amide or urea as linker between the hinge and DFG-out moieties. The NH group(s) of these linkers are involved as donor in hydrogen bond(s) with the side chain of the catalytically important Glu420 (Fig. 4A).[3] In contrast, the Glu420 side chain is far away from the aforementioned linker region in the C-helix-out conformer because Glu420 belongs to the C-helix (Fig. 4B) whose large displacement prevents the involvement of Glu420 as hydrogen bond acceptor.

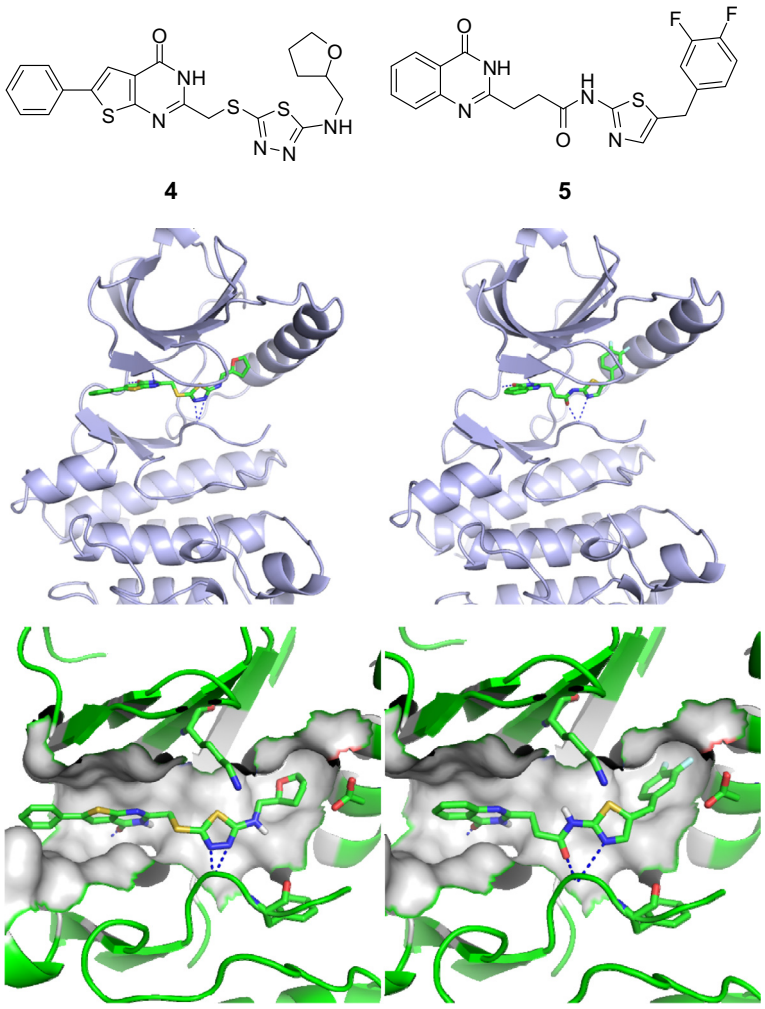

Figure 3. Chemical structure (top) and binding mode (bottom) of two novel Syk inhibitors identified by high-throughput docking into the DFG-in, C-helix-out conformation (3TUC) using the virtual screening procedure shown in Figure 1.

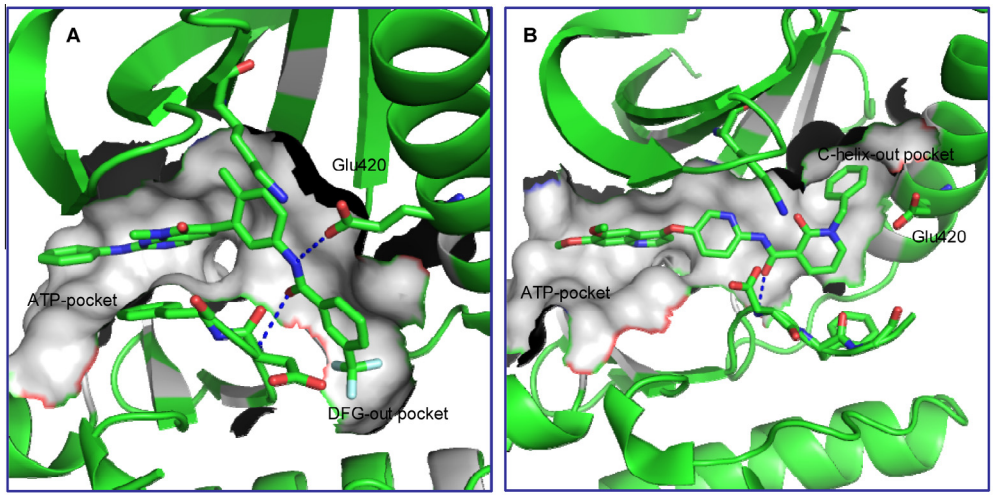

Figure 4. Comparison of binding to (A) the DFG-out conformation (inhibitor 3 of Ref. 5 in complex with Syk, PDB code 3TUD) and (B) the DFG-in, C-helix-out conformation (inhibitor **1** in complex with Syk, PDB code 3TUC).

However, the hydrogen bond with the backbone NH group of the DFG-motif Asp is maintained. Thus, the 'hybridization' approach originally proposed for type II DFG-out inhibitors[3] might also apply for C-helix-out inhibitors, for example, replacing the quinoline moiety of compound **1** with a different hinge-binding group from a type I inhibitor.

In summary, we have discovered two novel inhibitors of the Syk and ZAP70 kinases by high-throughput docking into a rare DFG-in, C-helix-out conformation of Syk. Compounds **4** and **5** are single-digit μM inhibitors of ZAP70 and JAK2. Their slightly higher potency for ZAP70 than Syk is likely to originate from the C-helix-out conformation being more populated by ZAP70 than Syk.

Acknowledgments

We are grateful to Armin Widmer (Novartis Basel) for the continuous support with the program WITNOTP, which was used for visual analysis. Calculations were performed on the Schrödinger compute cluster at the Informatikdienste of the University of Zurich. This work was supported financially by the Swiss National Science Foundation (grant to AC).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.01.083.

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