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Letter

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Combining Ligand- and Structure-Based Approaches for the Discovery of New Inhibitors of the EPHA2−ephrin-A1 Interaction

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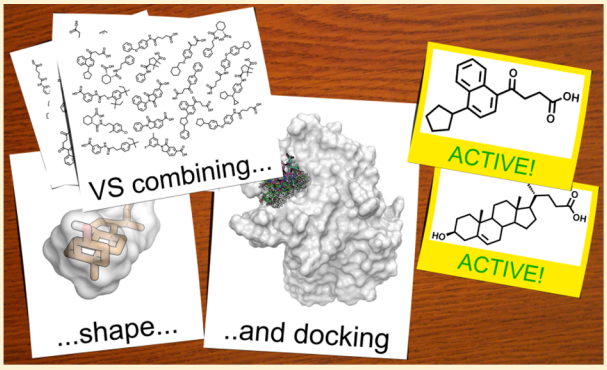
Supporting Information



ABSTRACT: The EPH receptor A2 (EPHA2) represents an attractive anticancer target. With the aim to identify novel EPHA2 receptor antagonists, a virtual screening campaign, combining shape-similarity and docking calculations, was conducted on a set of commercially available compounds. A combined score, taking into account both ligand- and structure-based results, was then used to identify the most promising candidates. Two compounds, selected among the best-ranked ones, were identified as EPHA2 receptor antagonists with micromolar affinity.

INTRODUCTION

The 14 erythropoietin-producing hepatocellular carcinoma (EPH) receptors represent the largest family of receptor tyrosine kinases and, together with their membrane-bound ligands, i.e. the ephrins, constitute a fundamental cell−cell communication system.[1] The EPH−ephrin system regulates most of the morphogenetic processes occurring during the embryonic development; whereas in the adult, it is mainly involved in the maintenance of cellular architecture in various epithelia and plays a key role in neural plasticity and regeneration of the nervous system.[2] Increasing evidence supports the notion that the EPHA2−ephrin-A1 system plays a crucial role in tumor and vascular functions during carcinogenesis.[3] Inhibition of the EPHA2 activity with monoclonal antibodies[4] or soluble receptors[5] has been recently shown to (i) arrest tumor growth, (ii) reduce the number of peripheral metastases, and (iii) disrupt tumor angiogenesis in animal models.[6] Altogether these findings propose EPHA2 as a target for the development of new antitumorigenic and antiangiogenic therapies.[7]

We have recently identified (3α,5β)-3-hydroxycholan-24-oic acid (lithocholic acid, LCA, **1**, Figure 1) as a competitive antagonist of the EPHA2 receptor during a high-throughput screening campaign conducted on a set of natural compounds.[8] On the basis of indications provided by docking of **1** into the ligand-binding domain of the EPHA2 receptor, we prepared a series of derivatives, which were able to disrupt EPHA2−ephrin-A1 interaction with potency in the low micromolar range.[9,10] Among them, the L-homo-tryptophan conjugate of **1** (UniPR129, Figure 1) emerged as the most promising EPHA2 antagonist, exhibiting submicromolar affinity for EPHA2 (K_i = 370 nM) and being capable of blocking angiogenesis in vitro at low micromolar concentration.[11] However, EPHA2 antagonists based on the 5β-cholan-24-oic acid scaffold suffer chemical and pharmacological limitations,[12] including (i) poor solubility, which so far has allowed only in vitro investigations, (ii) paucity of functional groups, which has hampered a significant expansion of the series,[9] and (iii) limited selectivity[13] due to the presence of a LCA-based moiety. Indeed, **1** is a physiological ligand of the G protein-coupled bile acid receptor TGR5[14] as well as of the nuclear targets farnesoid X receptor

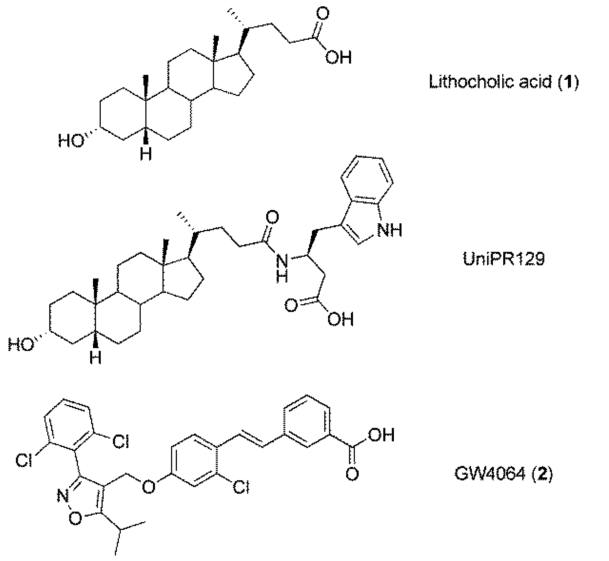


Figure 1. Chemical structures of selected EPHA2 receptor antagonists.

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(FXR)[15] and pregnane X receptor (PXR),[16] and it has been shown that some of these activities are also shared by its synthetic analogues.[13]

To search for novel and versatile chemotypes, we have recently applied a combined ligand- and structure-based screening approach on a set of FXR and TGR5 ligands and we identified GW4064 (**2**, Figure 1), a nonsteroidal FXR agonist, as a micromolar antagonist of the EPHA2 receptor.[17] Given the ability of a combined screening approach to identify novel EPHA2 receptor antagonists lacking the bile acid core, we here applied an in silico screening procedure based on a data fusion approach on a broader library of commercially available compounds.

METHODS

Database Preparation and Virtual Screening. Canvas 1.8[18] was used to manage the 110 597 two-dimensional (2D) structures of the Sigma-Aldrich online catalog, to calculate molecular properties (AlogP, MW) and to apply these properties as filters for compound selection. The 2D structures present in the filtered Sigma-Aldrich database (7,775 compounds with at least a carboxylic acid group) were converted into 3D structures using LigPrep 2.5[19] applying default settings for the generation of conformers and tautomers. Only the most abundant ionization state at pH 7.4 was modeled according to calculations performed with Epik 2.2. The database of 3D structures was submitted to shape-similarity screening[20] with Phase 3.3[21] using compound **1** in its docked conformation within EPHA2 as reference template. To this end, each 3D structure of the database was submitted to an *on-the-fly* conformational analysis with ConfGen which generated up to 100 conformers for each 3D structure. The overall database was ordered according to the shape-similarity score,[20] and only the best scored conformer for each entry was retained. A shape-similarity score of 0.5 was applied as a threshold for next step of the virtual screening protocol (i.e., the docking phase). Glide 5.7[22] was used to dock the resulting 3D-ligand structures into the EPHA2 ligand binding domain in standard precision (SP) mode. The obtained poses were rescored according to the extra precision (XP) scoring function, and only the 3D structures having a XP Glide Score lower than −5 kcal/mol were retained. Shape similarity score and XP Glide Score were combined in a single informative score called Z_{COMB} which was used to rank the resulting database. Compounds having a Z_{COMB} higher than 1 were submitted to cluster analysis (see the Supporting Information (SI)), and a represented set of compounds was selected for in vitro studies.

Chemistry. All the tested compounds were purchased from Sigma-Aldrich. They were identified by a combination of ^{1}H and ^{13}C NMR techniques (reported in the SI) and assayed for purity by HPLC/MS analysis resulting more than 95% pure.

Pharmacology. Disruption of the EPHA2−ephrin-A1 complex by small molecules was measured by means of an ELISA-based assay previously developed by our group[8] and described in the SI.

RESULTS AND DISCUSSION

The 2D structures of the compounds present in the Sigma-Aldrich catalog (110 597) were retrieved and submitted to the virtual screening funnel reported in Figure 2.[23]

The database was filtered taking into account known structure−activity relationships (SARs) around **1** and **2**. The

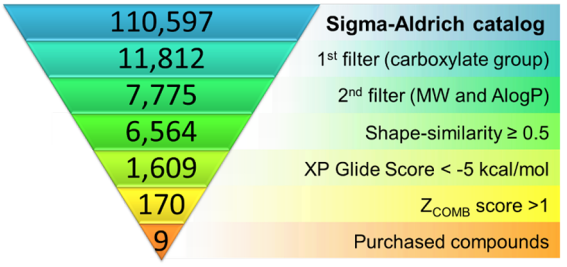

Figure 2. Virtual screening workflow applied in this study. The numbers reported in the funnel correspond to the number of unique compounds, excluding multiple tautomers stereoisomers and conformers.

carboxylic group of these compounds was found essential for the activity on EPHA2, as their corresponding methyl esters did not inhibit the formation of the EPHA2−ephrin-A1 complex up to 100 μM.[9,17] Thus, only compounds of the Sigma-Aldrich database bearing one or more carboxylic groups were retained. The resulting data set, which contained 11 812 2D structures, was submitted to a second filtering procedure aimed at retaining only those molecules with molecular weight (MW) and lipophilicity (AlogP) centered around those of **1** and **2** (200−600 for MW and 0−8 for AlogP). The application of these filters further reduced the size of the data set to 7775 2D structures. Ligprep[19] was then used to generate 3D structures of all the 2D entries present in the filtered database, using default settings for the generation of conformers, tautomers and stereoisomers. The resulting database was subsequently applied to perform ligand- and structure-based analyses. A shape-similarity score[20] (equal to one for identical molecules and to zero for dissimilar structures) was then calculated between each 3D structure of the database and **1** in its docked conformation within the EPHA2 receptor[9] (Figure 3) using Phase software.[21]

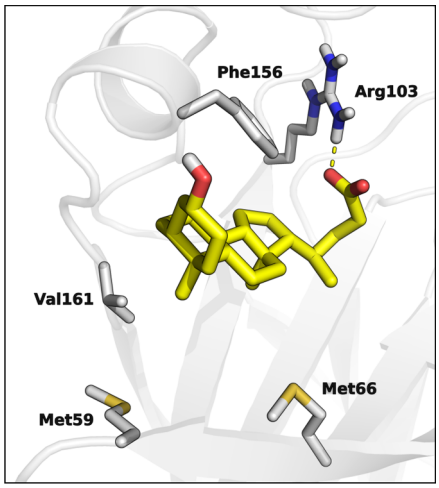

Figure 3. Docked conformation of **1** (yellow carbons) into EPHA2 receptor.[9,17]

Shape-similarity scores ranged from 0.863 to 0.233, suggesting the presence of highly dissimilar chemotypes within the compounds data set. Since compound **2** is characterized by one of the lowest similarity score to **1** among EPHA2 receptor antagonists,[17] we hypothesized that compounds characterized by similar or higher shape-similarity scores might possess the structural features required to disrupt the EPHA2−ephrin-A1

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assembly. Accordingly, molecules showing a shape-similarity score being similar or higher than that of compound **2** (i.e., 0.5) were retrieved and docked into the EPHA2 receptor using Glide in Standard Precision (SP) mode. The resulting poses were subsequently rescored applying the Extra Precision (XP) scoring function.[24] Docking results showed that almost all the compounds were accommodated into the EPHA2 binding cavity with an arrangement similar to that of **1** (Figure 3). Despite the similar orientation into the EPHA2 receptor, the compounds were characterized by highly dissimilar XP Glide Scores, which probably reflect significant differences in their ability to establish productive interactions into the binding site. In particular, docking scores were likely to be strongly governed by the electrostatic interaction between the ligand carboxyl acid group and the protonated side chain of Arg103. All compounds showing a XP Glide Score lower than that of **1** (i.e., −5 kcal/mol) were retained as they might exhibit features required to bind EPHA2.

To identify the most promising candidates among the selected compounds (1609), a combined score, taking into account the results of both shape screening and docking simulations, was calculated and used as a final filter. Since the performances of different screening methods are not known a priori, the application of a single ligand- or structure-based screening protocol may hamper the identification of some classes of active compounds. For this reason, results of multiple screening approaches can be combined into a unique score, which can allow for the strengths of a method to balance the limitations of others.[25,26] To this end, shape-similarity score and XP Glide score were converted in standard scores called Z_{SHAPE} and Z_{DOCK}, respectively, and then averaged in one single descriptor named Z_{COMB} (see the SI for details). Compounds showing high Z_{COMB} scores resided among the top-ranked molecules in both shape screening and docking simulations and can be thus considered as the most promising candidates (Figure 4). Here, compounds showing a Z_{COMB} score higher than 1 were extracted from the data set (170 unique structures) and analyzed further.

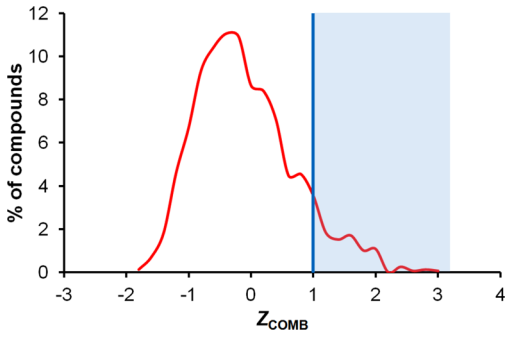

Figure 4. Distribution of the Z_{COMB} scores at the end of the virtual screening procedure.

A cluster analysis based on MOLPRINT2D fingerprints was subsequently performed on these unique structures to identify the most representative chemical scaffolds. Six different clusters were identified, three of which (clusters A, B, and C) were singletons corresponding to purvalanol B (**3**, Figure 5), bexarotene (**4**, Figure 5), and 3β-hydroxy-Δ^5-cholenic acid (**5**, Figure 5).

Compound **3** is a potent inhibitor of cyclin-dependent kinases (CDKs)[27] and was considered not interesting for our research. **4** is a retinoid X receptor (RXR) agonist structurally related to the stilbene carboxylic acid derivative TTNPB (Figure 5), a compound that already proved to be inactive on the EPHA2 receptor.[18] Due to its high similarity to an inactive molecule, compound **4** was excluded from the subset of potential EPHA2−ephrin-A1 inhibitors. **5** is a steroidal compound structurally related to the class of the oxysterols, which are natural agonists of the liver X receptor (LXR).[28] Since **5** showed the highest shape-similarity value (0.863) and the highest Z_{COMB} score (2.969) among all the clustered compounds, it represented one of the most promising candidates and was thus selected for in vitro experiments. A set of compounds was also selected among clusters D, E, and F. To this aim, structures belonging to these clusters were ranked according to the Z_{COMB} score, and the best-scored ones were inspected on the basis of their ability to establish the highest number of interactions within the binding site of the EPHA2 receptor. This analysis identified eight compounds (**6**−**13**) as the most promising candidates of clusters D−F (Figure 6).

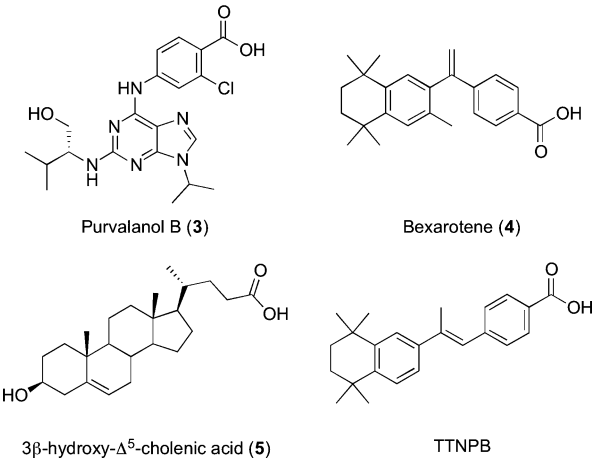

Figure 5. Chemical structures of singletons **4**−**6** identified after cluster analysis. The structure of TTNPB is depicted to highlight its similarity to compound **4**.

The final set of compounds purchased for experimental testing is thus composed by **5**−**13**. Compounds **5**−**13** were tested for their ability to inhibit EPHA2−ephrin-A1 association by an ELISA assay. While **5** and **6** were able to significantly prevent ephrin-A1 binding at 50 μM concentration, **7**−**13** were inactive or poorly active (i.e., 20% of inhibition for **12**) in the same conditions (Figure 7A).

Given the ability of compounds **5** and **6** to block EPHA2−ephrin-A1 association, their potencies were evaluated through dose−response curves. Both compounds dose-dependently inhibited ephrin-A1 binding to the EPHA2 receptor, showing a half-maximum inhibitory concentration (IC_{50}) of 32 and 67 μM, respectively (Figure 7B).

Visual inspection of docking results showed that compounds **5** and **6** are able to mimic the crystallized conformation of the so-called "G−H loop" of ephrin-A1, a highly conserved region of ephrin ligands fundamental for binding to the EPHA2 receptor.[29] In particular, their carboxylate group well superposed the backbone carbonyl group of Thr112 of ephrin-A1, mimicking its hydrogen-bond interaction with the Arg103 side chain. The A-ring of the steroidal nucleus of **5** and the cyclopentyl ring of **6** occupied the same positions of the lipophilic side chains of Leu116 and Phe114 of the ephrin-A1, respectively (Figure 8).

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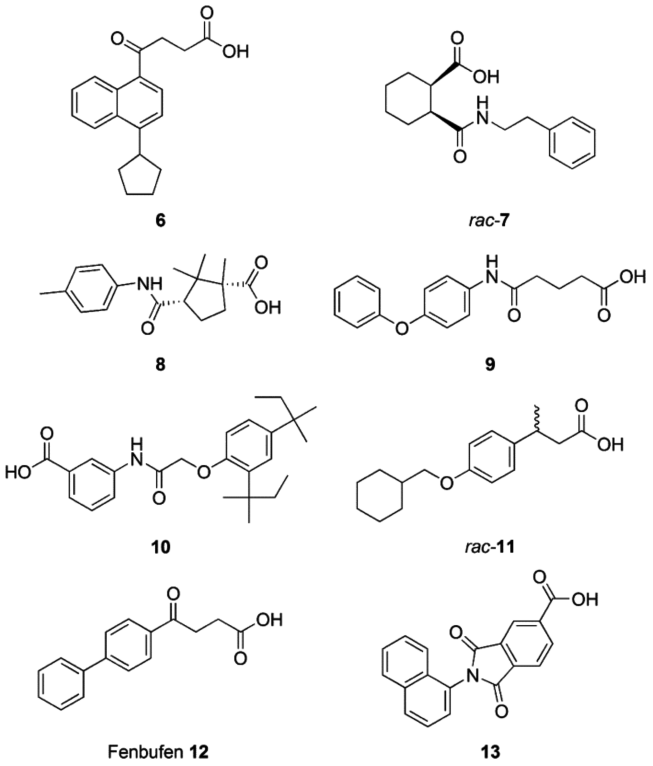

Figure 6. Chemical structures of compounds **6**–**13**.

It is interesting to note that, shape-similarity scores and docking scores obtained for compounds **5**–**13** were not correlated (Table S1), with the former being able to efficiently recognize **5** and **6** as the active derivatives. Indeed, **5** and **6** were the two best-ranked molecules according to the shape-similarity. On the other hand, compounds **5** and **6** were not ranked within the top 10% of the ordered lists obtained from docking simulations. In this case, application of docking score as a unique criterion for selection would have led to discard compounds **5** and **6** from the list of candidates. Interestingly, in our previous study,[17] the active derivative **2** was correctly recognized by docking but was one of the worst-ranked by shape-similarity. In such a scenario, when the performances of different virtual screening approaches cannot be predicted a priori, the data fusion technique, despite affected by some limitations (i.e., accuracy of scoring functions and statistical distribution of scores),[20] represents a valuable strategy to identify active compounds[26,27] However, it is worth mentioning that the application of protocols able to take into account protein flexibility, such as induced-fit docking and molecular dynamics simulations, might improve the screening results as they could favor a mutual adaptation of the binding site residues around the ligand molecule, avoiding the formation of unfavorable ligand–receptor contacts that would otherwise have a negative effect on the docking score.

CONCLUSIONS

We described the application of a combined in silico protocol based on shape-similarity calculations and docking experiments to identify new inhibitors of the EPHA2–ephrin-A1 interaction among a large set of commercially available acid compounds. A data fusion technique was applied to combine the results of ligand- and structure-based methods into a single score, which was subsequently used to rank the molecules data set. Among the best-ranked compounds, the Δ^5-cholenic acid **5** and the 4-(4-cyclopentylnaphthalen-1-yl)-4-oxobutanoic acid **6** were identified as disruptors of the EPHA2–ephrin-A1 interaction. These two compounds represent novel chemotypes, which may lead to effective EPHA2 antagonists with improved chemical and/or pharmacological properties. Although compound **5** directly derives from a class of potent LXR agonists (i.e., the oxysterols), it does not affect cellular responses mediated by LXR functioning (i.e., efflux of cholesterol in macrophages, Figure S1) up to 30 μM. Moreover, Δ^5-cholenic acid derivatives fail to activate physiological targets of LCA (**1**) including FXR and PXR receptors,[30] suggesting that it is possible to achieve EPHA2 selectivity working around the Δ^5-cholenic acid nucleus.

As opposed to **1**, **6** is characterized by a lower affinity for the EPHA2 receptor. That said, **6** exhibits a higher ligand efficiency value (0.26 kcal/mol/atom) with respect to both **1** (0.21 kcal/mol/atom) and **5** (0.23 kcal/mol/atom) and is characterized by a more chemically versatile scaffold, which should ease a rather efficient exploration of the SARs and a further expansion of the chemical series.

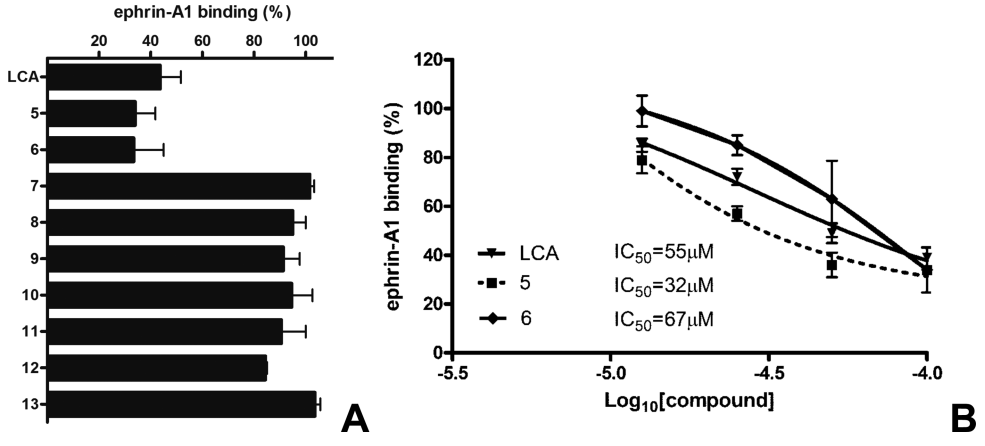

Figure 7. (A) Percentage of ephrin-A1 binding to EPHA2 receptor after coincubation with compounds **5**–**13** at 50 μM concentration. LCA (**1**) at 50 μM has been used as a control. (B) Dose–response curves for compounds LCA (**1**), **5**, and **6** toward EPHA2–ephrin-A1 interaction.

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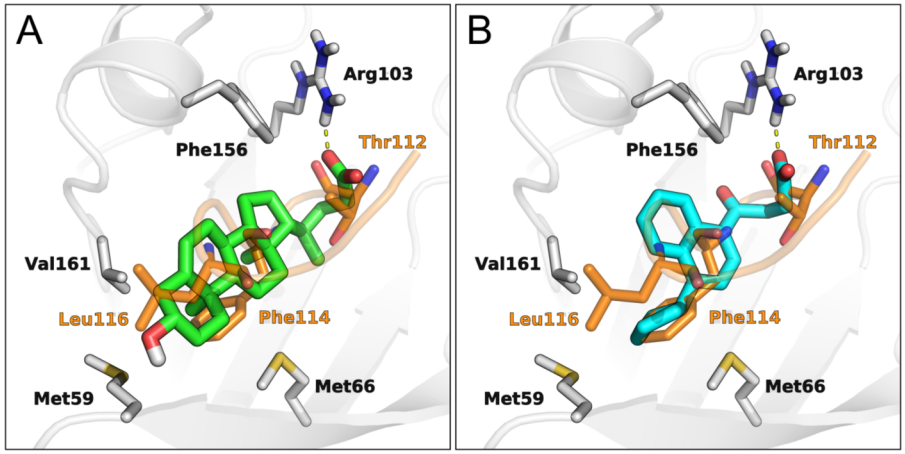

Figure 8. Best-docking poses obtained for compounds **5** (A, green carbons) and **6** (B, cyan carbons) within the EPHA2 receptor binding site (light gray). The Thr112-Glu119 sequence of the cocrystallized G−H loop of ephrin-A1 (orange) is reported in orange.

■ ASSOCIATED CONTENT

Supporting Information

Details of docking simulations, shape-similarity calculations, and cluster analysis. XP Glide Scores and shape-similarities of compounds **5**−**13**. NMR spectra of compounds **5**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EPHA2, ephrin receptor A2; FXR, farnesoid X receptor; TGR5, G protein-coupled bile acid receptor 1; SP, Standard Precision; XP, Extra Precision; LXR, liver X receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; ELISA, enzyme-linked immunosorbent assay

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