Bioorganic & Medicinal Chemistry Letters 19 (2009) 2965–2968

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Spiroimidazolidinone NPC1L1 inhibitors. 1: Discovery by 3D-similarity-based virtual screening

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ARTICLE INFO

Article history:
Received 19 February 2009
Accepted 14 April 2009
Available online 17 April 2009

Keywords:
Virtual screening
NPC1L1 inhibitors
Cholesterol absorption inhibitors
Superposition
Molecular modeling

ABSTRACT

A series of spiroimidazolidinone NPC1L1 inhibitors was discovered by virtual screening of the Merck corporate sample repository using 3D-similarity-based screening. Selection of 330 compounds for testing in an in vitro NPC1L1 binding assay yielded six hits in six distinct chemical series. Follow-up 2D similarity searching yielded several sub- to low-micromolar leads; among these was spiroimidazolidinone **10**, with an IC_{50} of 2.5 μM. Compound **10** provided a useful scaffold to initiate a medicinal chemistry campaign.

Hypercholesterolemia represents a major health risk for millions of people and is associated with increased rates of heart attack, stroke, and other cardiovascular disorders.[1] Along with statins, which lower LDL cholesterol levels by inhibition of cholesterol biosynthesis mediated by HMG–CoA reductase, the cholesterol absorption inhibitor ezetimibe has been shown to be effective in decreasing blood LDL cholesterol levels by decreasing uptake of dietary cholesterol.[2]

Ezetimibe was discovered using an empirical, in vivo screening protocol.[3] It was later determined that ezetimibe inhibits intestinal cholesterol absorption by binding to NPC1L1 (Niemann–Pick C1-like protein 1), a member of the RND permease family of transporters,[4,5] and recently two residues on loop C of NPC1L1 were shown to be important for ezetimibe binding.[6] The development of an in vitro binding assay[4,5] has considerably increased the capacity for testing compounds, suggesting the possibility of identifying structurally novel NPC1L1 inhibitors.

Ezetimibe is a 2-azetidinone with three distal aryl rings—one phenol and two *p*-fluoro-phenyl groups. In vivo, the phenol undergoes glucuronidation to yield the more potent NPC1L1 inhibitor ezetimibe–glucuronide (**2**).[7]

As part of a project aimed at discovering new NPC1L1 inhibitors, we were interested in pursuing chemical scaffolds other than that of ezetimibe. Although slight modifications to the 2-azetidinone core have been reported,[8–10] we were interested in finding a more diverse set of new chemical scaffolds. We therefore decided to pursue virtual screening to select compounds from our sample repository for testing in the benchtop medium-throughput NPC1L1 binding assay.

Here, we report on the discovery of a diverse set of non-β-lactam NPC1L1 inhibitors using a 3D-similarity-based virtual screen of the Merck corporate sample repository followed by 2D similarity searching. One of these series was selected for medicinal chemistry optimization.

Virtual screening. Because we were interested in obtaining novel chemical scaffolds, we decided to use 3D virtual screening methods in place of 2D similarity techniques for the initial screen.[11] High-throughput docking was not possible because crystal structures are available neither for NPC1L1 nor for close homologs; therefore, we chose 3D-similarity-based virtual screening and pharmacophore searches. Ligand-based 3D virtual screening has been successfully used to 'scaffold hop', that is to identify leads with a similar shape despite having a different chemical core.[12,13] The Merck proprietary superposition program SQ[14] has been shown to provide good enrichment in virtual screens of a number of targets.[11] SQ combines shape and electrostatic/hydrogen-bonding information and therefore can be considered a 3D similarity method. Non-hydrogen atoms in the molecules are assigned as one of seven atom types: cation, anion, hydrogen-bond donor, hydrogen-bond acceptor, polar, hydrophobic and 'other'. The SQ score is calculated by optimization of an objective function which takes

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doi:10.1016/j.bmcl.2009.04.031

into account the similarity of the types of each atom and the distance between them. The score reflects the quality of the superposition between a query molecule and database entries. Users can specify required or preferred matching centers in order to upweight certain chemical features inferred from known SAR.

A recently rewritten version of the program, SQW, uses a Dice-like normalized version of the score:

$$SQ_{\mathrm{norm}}(A,B) = \frac{2 \times SQ_{\mathrm{raw}}(A,B)}{SQ_{\mathrm{raw}}(A,A) + SQ_{\mathrm{raw}}(B,B)},$$

where $SQ_{\mathrm{norm}}(A,B)$ is the normalized score between molecules A and B, $SQ_{\mathrm{raw}}(A,B)$ is the raw score between A and B, and $SQ_{\mathrm{raw}}(A,A)$ and $SQ_{\mathrm{raw}}(B,B)$ are the raw scores of A with itself and B with itself, respectively. This normalization corrects for the size bias intrinsic to the raw score, wherein larger compounds are typically scored better, and therefore allows one to better rank truly similar compounds in a database search.

We used two separate probe molecules to screen the sample repository: ezetimibe–glucuronide (**2**) and the more potent analog **3** (Fig. 1) that is substituted with a sulfonamide-acetylene linker on the *N*-phenyl ring.[15] Because the bioactive conformation of ezetimibe is unknown, we performed conformational analysis on each of the molecules to find low-energy conformations to construct the probe. As expected based on the rigid nature of the azetidinone ring, the conformations differed primarily in the 3-phenylpropyl chain. Two diverse conformations of this chain were selected.

Two separate 3D similarity searches were performed. One of the searches used **3** as probe, and the second used **2**; a different propyl sidechain conformation was used for each probe. The two screens also differed in the definition of the 'essential points'.[14] In the first screen, using **3** as probe, none of the atoms were considered essential, while in the second screen, five atoms were marked as essential and upweighted accordingly. The much higher potency of **2** relative to **1** suggested that the glucuronide is an important binding element, so we marked one of the glucuronide hydroxyl groups as essential. Similarly, the hydroxyl group on the propyl chain was marked essential due to its large effect on NPC1L1 activity.[3] Finally, the spatial arrangement of the aromatic rings was also considered likely to be an important recognition element; therefore, the three aromatic rings were also upweighted in the second search by marking one carbon atom as essential.

A total of approximately 1 million compounds were scored in each screen, and for each compound up to 25 pre-computed conformations were considered, with a total of 20 million conformers scored. The top-scoring compounds from each of the two 3D-similarity-based searches were inspected visually. A total of 2667 compounds were inspected, of which 183 were selected for experimental binding determination on the basis of their perceived chemical tractability and drug-likeness, as well as the quality of the overlay.

Despite the fact that our set of lead compounds was limited to a single chemical series, we decided to augment our SQW search with a pharmacophore search because of the general rigidity of the azetidinone core, and because examination of the patent literature suggested functionality important for NPC1L1 binding affinity. Pharmacophore searching was performed using MOE software (Chemical Computing Group, Montreal).

Two separate pharmacophore searches were performed. The searches used a seven-feature pharmacophore, including all three aryl groups, the glucuronide, the azetidinone carbonyl H-bond acceptor, and polar features for the hydroxyl on the propyl side chain and the sulfonamide of **3**. Due to the flexibility of the propyl chain of **1–3**, the pendant aryl ring was represented with a larger pharmacophore feature than the other two aryl rings. The glucuronide and sulfonamide features were also slightly larger due to rotational freedom around the O-glucuronide and alkynyl bonds, respectively. The first search required all seven features to match but allowed any polar group to match the glucuronide, while the second search required an acid to match the glucuronide feature but required only six of the seven features to constitute a match. From the pharmacophore searches, 147 compounds were selected for binding assay testing.

The 330 compounds selected from the 3D-similarity and pharmacophore searches were tested first at a single concentration, and compounds displaying >50% inhibition at 100 μM were titrated to determine the IC_{50}. Binding was determined by displacement of ^{35}S-labeled **3** from either enterocyte brush border membranes, or from membrane preparations of HEK-293 cells transiently expressing recombinant NPC1L1. This assay is a modification of the original method developed using ^{3}H-ezetimibe-glucuronide,[4,5] and will be described in detail elsewhere.[15] Six out of the 330 compounds displayed an IC_{50} below 100 μM, corresponding to a hit rate of 1.8%. The hits were from six different chemical series and are shown in Figure 2. The SQW-derived overlay between probe **3** and virtual screening hit **5** is shown in Figure 3.

The active compounds shown in Figure 2 all have very low similarity to the probe compounds using 2D descriptors, and therefore would not have been identified by 2D-based similarity searching techniques. The closest match was between **2** and **4**, with an atom-pair (AP)[16] Dice similarity of 0.35; AP similarities to the probes were below 0.3 for the other five hits.

All of the hits were derived from the 3D-similarity-based screens; the pharmacophore searches did not yield any actives. This is likely to be due at least in part to the fact that the lack of structurally diverse leads and the limited available SAR greatly hampered the generation of a pharmacophore hypothesis. As a result, unimportant features are likely to have been included, resulting in hits that matched all features but did not have high overall resemblance to the known actives. For example, although a carboxylic acid was considered to be an important binding element based on the relative potencies of **1** and **2**, three of the six hits, including the two most potent, have no acidic functionality.

Of the six hits from 3D-similarity searches, four (**4**, **5**, **6**, and **8**) came from the search using **3** as probe and lacking 'essential points'; the other two hits (**7** and **9**) came from the search performed with **2** and essential points. However, because more com-

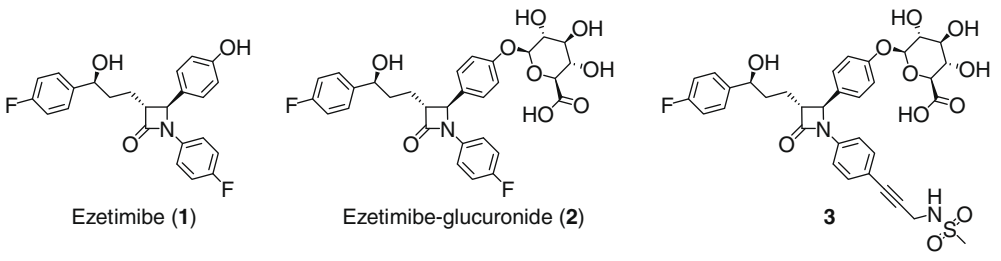

Figure 1. Structures of 2-azetidinone NPC1L1 inhibitors.

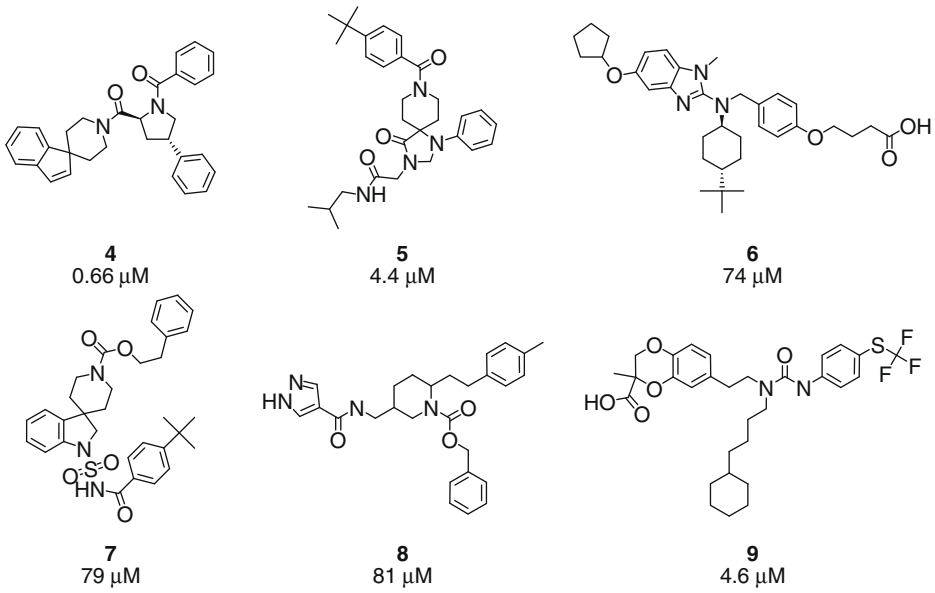

Figure 2. Inhibitors of human NPC1L1 identified by 3D-similarity-based virtual screening.

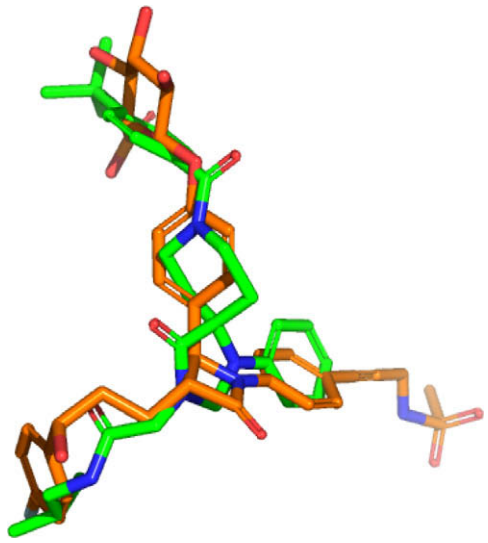

Figure 3. Overlay of **3** (orange) and screening hit **5** (green) as identified by the 3D-similarity-based screening software SQW.

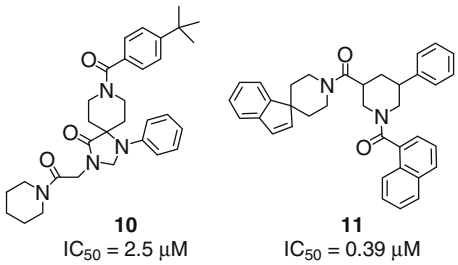

Figure 4. NPC1L1 inhibitors identified by 3D-similarity-based virtual screening followed by 2D similarity searches.

pounds were selected from the search based on **3**, the hit rates were actually very similar, 2.8% for the **2**-based search versus 3.4% for the **3**-based search.

Compounds **4** and **5** were members of combinatorial libraries and therefore had a number of analogs in the sample collection with the same scaffold. To follow up on the original hits, therefore, 2D similarity searches were performed using the Merck proprietary program TOPOSIM.[17] A total of 590 compounds were selected for screening. Two of the most active leads identified in this round of screening are shown in Figure 4. Among the most attractive leads was the spiroimidazolidinone **10**, with a binding affinity for human NPC1L1 of 2.5 μM.

In order to determine whether the binding affinity would translate into functional activity, **10** would need to be tested in an in vivo cholesterol absorption assay. The affinity of **4** and **10** for NPC1L1 in various rodent species was therefore tested to determine its binding affinity. Table 1 shows the results obtained for three rodent and three additional animal species. Whereas the azetidinone inhibitors have similar potency for human NPC1L1 and the orthologs tested, the screening leads showed a substantially greater potency for human NPC1L1. Commensurate with the selectivity of **10** for human NPC1L1 compared with other species, it did not inhibit in vivo cholesterol absorption in rodent. The in vivo cholesterol absorption assay measures the uptake of radiolabelled cholesterol in a rodent following administration in an animal in the presence or absence of test compound. The compounds were predosed orally in C57BL/6 male mice in a 0.2 mL 0.25% methocel suspension. Thirty minutes later, the mice were orally dosed with a 0.2 mL mixture of tritium-radiolabeled cholesterol in IntraLipid. After 1.5 h or 5 h, the mice were euthanized and weighed. The blood was collected by cardiac puncture, and the plasma ^{3}H-cholesterol was measured. The liver was collected, weighed, and saponified, and the liver ^{3}H-cholesterol was measured.

Medicinal chemistry effort was initiated to improve the binding affinity of spiroimidazolidinone **10** on both human NPC1L1 and that of other preclinical species in order to demonstrate in vivo efficacy of these novel structures with binding affinity to NPC1L1. The results of these SAR studies will be described in a separate Letter.[18]

Conclusion. Using 3D-similarity-based virtual screening followed by 2D similarity searches, we have identified several compounds with diverse, non-azetidinone scaffolds displaying low- or sub-micromolar binding affinity to human NPC1L1. These compounds had much lower binding affinity to NPC1L1 from other

Table 1
Binding affinity of azetidinone inhibitors **2** and **3** and virtual screening leads **4** and **10** to human NPC1L1 and to NPC1L1-containing enterocyte brush border membranes (BBM) from various species

	IC_{50} (μM)							
Compound	Human NPC1L1	Human BBM	Rat BBM	Hamster BBM	Guinea pig BBM	Dog BBM	Rhesus BBM	Pig BBM
2	0.3	0.3	0.58	0.65	3.3	0.06	0.03	0.32
3	0.007	0.02	0.016	0.022	0.071	0.008	0.02	0.013
4	0.66	5.3	34	62	118	>500	146	>500
10	2.5	10.0	110	41	470	170	77	>500

species, in contrast to ezetimibe, for which the affinity for human and nonhuman NPC1L1 is similar. Consistent with this human selectivity, **10** did not show activity in a rodent in vivo cholesterol absorption assay. However, the leads served as attractive starting points for a medicinal chemistry program.

Acknowledgments

The authors thank Alastair Hill and Joseph Shpungin for help with the pharmacophore search.

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