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Aminopyrrolidineamide inhibitors of site-1 protease

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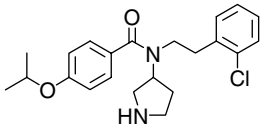
Abstract—The discovery and efficacy of a series of potent aminopyrrolidineamide-based inhibitors of sterol regulatory element binding protein site-1 protease is described.

Site-1 protease (S1P) is a unique membrane-bound serine protease responsible for cholesterol-sensitive cleavage of the transcription factor sterol regulatory element binding protein (SREBP).[1–4] The S1P-mediated SREBP cleavage is the first of two cleavage events required to release mature transcription-competent SREBP from the Golgi and enable its passage to the nucleus[1–3] where it regulates the gene expression of a wide variety of lipogenic genes involved in cholesterol synthesis (e.g., HMG-CoA synthase, HMG-CoA reductase, squalene synthase, etc.) and fatty acid synthesis (e.g., fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, etc.) as well as the expression of a variety of other genes involved in nutrient uptake and processing, such as the LDL receptor.

Inhibition of S1P activity has the potential to simultaneously inhibit both the cholesterolgenic and fatty acid synthetic pathways and, as a consequence, lower plasma cholesterol and triglycerides in patients with dislipidemia.[1–3] A S1P inhibitor would thus combine the benefits of an HMG-CoA reductase inhibitor[5] with those of an acetyl-CoA carboxylase inhibitor[6] and therefore would be useful not only for patients with dislipidemia, but also for those with a variety of cardiometabolic risk factors associated with insulin resistance, diabetes, obesity, and the metabolic syndrome.[6,7] With these potential therapeutic benefits in mind, we initiated a program to look for small molecule inhibitors of S1P. Herein, we describe the initial results of this effort.

A high throughput screen, using purified human S1P and a fluorescent assay format that assesses cleavage of the synthetic cleavage site peptide, Ac-VFRSLK-MCA (360 nm excitation; 460 nm emission),[8] was employed to search the Pfizer compound file for S1P inhibitors. Only a handful of compounds showed greater than 50% inhibition of S1P at 2.25 μM. One of the most interesting hits was the aminopyrrolidine derivative **1a** (Fig. 1), with an IC_{50} of 1.4 μM. This compound was originally produced as part of a large (3786 compounds) library, and an analysis of the high throughput screen results revealed that 39 of these library compounds inhibited S1P greater than 25% at 2.25 μM. Retesting these compounds yielded measurable IC_{50}s for two close-in derivatives, **1b** and **1c** (Table 1). All of the other library compounds had IC_{50}s greater than 30 μM.



1a

Figure 1. High throughput screening hit **1a**.

Keywords: Site-1 protease; SREBP; Sterol regulatory element binding protein; Metabolic syndrome.
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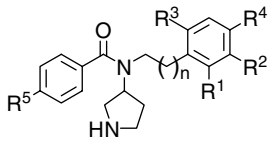
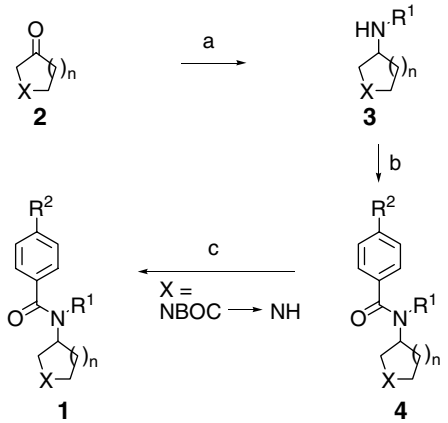
Table 1. S1P IC_{50} values for compounds **1a–n**



Compound	*n*	R^1	R^2	R^3	R^4	R^5	S1P IC_{50} (nM)
1a	1	Cl	H	H	H	O^{i}Pr	1400
1b	1	F	H	H	H	O^{i}Pr	9000
1c	1	H	Cl	H	H	O^{i}Pr	9600
1d	1	OMe	H	H	H	$CH_2N(Et)_2$	540
1e	1	OMe	H	H	H	OBn	310
1f	1	Cl	H	H	H	$CH_2N(Et)_2$	840
1g	1	Cl	H	H	H	OBn	590
1h	0	Cl	H	H	H	O^{i}Pr	>5000
1i	1	Cl	H	Cl	H	$CH_2N(Et)_2$	430
1j	1	H	H	H	Cl	$CH_2N(Et)_2$	3700
1k	1	H	Cl	H	H	OBn	510
1m	1	Me	H	H	H	$CH_2N(Et)_2$	1600
1n	1	OEt	H	H	H	$CH_2N(Et)_2$	16,000

Follow-up libraries around compound **1a** were initiated. The first library was a full combinatorial cross of 11 amines by 32 acids, with monomers chosen based on similarity to the original hit and some of the SAR gleaned from the initial high throughput screen. The core template remained static. The library was prepared as shown in Scheme 1, involving reductive amination of the BOC-pyrrolidinone followed by amide coupling and removal of the BOC protecting group. A total of 243 out of 352 (69%) compounds were returned after synthesis and HPLC autopurification. Potencies of selected compounds are listed in Table 1. Several compounds were significantly more potent than the initial lead, including the dibasic amines **1d** and **1f**, and the more lipophilic compounds **1e** and **1g**. The 2-substituted phenethylamine monomer was important for S1P activity, 2-Cl and 2-methoxyphenethylamine derived compounds had comparable potency, with the 2-methylphenethylamine (**1m**) slightly less potent.

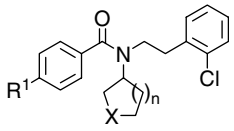


Scheme 1. Synthesis of S1P inhibitors. Reagents: (a) i—R^1NH_2, EtOH, Toluene; ii—$NaBH_4$, EtOH; (b) substituted benzoic acid, HBTU, DIPEA, DCE; (c) HCl, DCE.

Increasing the size of the 2-substituent was detrimental, exemplified by the ethoxy compound **1n**. In general, 2-substituted phenethylamines led to equal or better potency relative to 3-substituted phenethylamines (**1a** vs **1c** and **1g** vs **1k**) while 4-substituted phenethylamines led to significantly less active compounds (**1j** vs **1f**). Additional substitutions on either phenyl ring were detrimental, the lone exception being the 2,6-dichlorophenethylamine monomer in **1i**. The 2-carbon chain in the phenethylamine was also critical, as the benzylamine derivative **1h** had no measurable activity. Compounds with amides other than 4-substituted benzamides also had no measurable activity with any of the phenethylamine monomers. Based on the data from this initial library we felt that there might be an opportunity for further potency improvements by additional modification of the benzamide substituent at the 4-position.

A second library was prepared using the route shown in Scheme 1 in which the 4-isopropoxybenzamide and 2-Cl-phenethylamine monomers were kept constant while the core was varied. No measurable S1P inhibition was observed with any of these compounds. Selected data are shown in Table 2, including the tetrahydrothiophene

Table 2. S1P IC_{50} values for compounds **1o–1t**



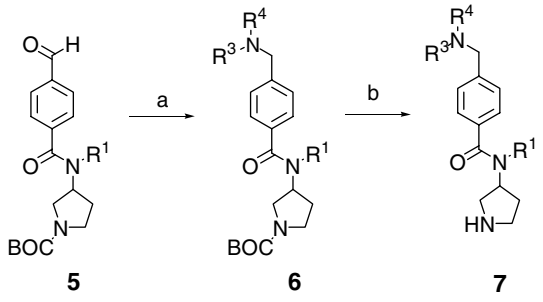
Compound	X	*n*	R^1	IC_{50} (nM)
1a	NH	1	O^{i}Pr	1400
1o	NCH3	1	O^{i}Pr	>30,000
1p	S	1	O^{i}Pr	>30,000
1q	NCH=O	1	O^{i}Pr	>30,000
1r	NH	2	O^{i}Pr	>30,000
1s	NH	0	O^{i}Pr	>30,000
1t	O	1	Methyl(1-piperidine)	>30,000

1p, *N*-methylpyrrolidine **1o**, *N*-formylpyrrolidine **1q**, 3-piperidine **1r**, and azetidine **1s**. A tetrahydrofuran analog **1t** was synthesized at a later date and also had no measurable inhibition of S1P.

A third library was then synthesized to follow up on the activity of the diethylamine derivatives **1d** and **1f**. The template formation involved reductive amination of BOC-pyrrolidinone with 2-(2-methoxyphenyl)ethylamine or 2-(2-chlorophenyl)ethylamine and amide formation with 4-carboxybenzaldehyde (Scheme 1), followed by parallel chemistry (reductive amination and BOC protecting group removal, Scheme 2) for the conversion to the final diamines. Many of these compounds showed significant potency improvements (Table 3), **7b** was the most potent compound from this library with an IC_{50} of 8 nM.

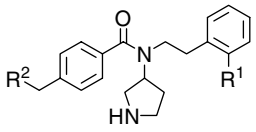
Compound **1d** was selected for further evaluation because it had reasonable potency and lower molecular weight and lipophilicity relative to the other potent compounds. The enantiomers were separated by preparative HPLC,[9] and the more potent **1u** (PF-429242, Table 4) was assigned the R stereochemistry after direct synthesis from the chiral **8** (Scheme 3), followed by amide coupling and removal of the BOC protecting group as in Scheme 1.

PF-429242 showed a high degree of selectivity for S1P inhibition relative to other serine proteases, exhibiting an IC_{50} for S1P inhibition of 170 nM and showing no significant inhibition of trypsin, elastase, proteinase K, plasmin, kallikren, factor XIa, thrombin, or furin at concentrations up to 100 μM and only modest inhibition of urokinase (IC_{50} = 50 μM) and factor Xa (IC_{50} = 100 μM). In cultured human liver (Hep-G2) cells, PF-429242 prevented proteolytic processing and nuclear translocation of SREBP[10] (complete inhibition at a dose of 10 μM), reduced the expression of key genes involved in cholesterol synthesis (e.g., HMG-CoA synthase; EC_{50} = 0.3 μM) and fatty acid synthesis (e.g., fatty acid synthase; EC_{50} = 2 μM),[11] and inhibited both pathways (cholesterol synthesis, EC_{50} = 600 nM; fatty acid synthesis, 43% inhibition at 10 μM)[12] in the absence of any cytotoxicity (LDH release assay). LDL receptor gene expression and LDL receptor-mediated LDL internalization[13] by Hep-G2 cells were also inhibited by PF-429242, but to a very small degree relative to inhibition of cholesterol synthesis. For example, LDL receptor-mediated LDL internalization was not altered by PF-429242 at concentrations that produced 65% inhibition of cholesterol synthesis, and was only inhibited by



Scheme 2. Synthesis of dibasic S1P inhibitors. Reagents: (a) R^3R^4NH, $N(CH_3)_4BH(OAc)_3$, DCE; (b) HCl, DCE.

Table 3. IC_{50}s for selected second iteration compounds **7a**–**h**



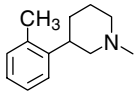
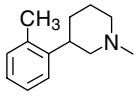
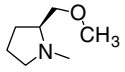
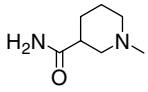
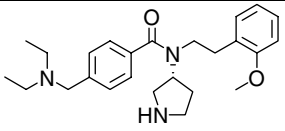
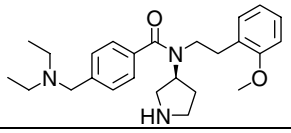
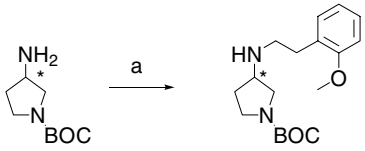
Compound	R^1	R^2	IC_{50} (nM)
7a	OMe		37
7b	Cl		8
7c	OMe		29
7d	OMe		95
7e	OMe		100
7f	OMe		250
7g	OMe		140
7h	OMe		160

Table 4. IC_{50}s and enantiomeric configurations of **1u** and **1v**

Compound	Structure	IC_{50} (nM)
1u (PF-429242)		170
1v		970



Scheme 3. Synthesis of chiral 3-pyrrolidineamides. Reagents: (a) 1-(2-bromoethy)-2-methoxybenzene, Et_3N.

35% at concentrations that inhibited cholesterol synthesis by >90%.

PK data for PF-429242 showed that it had rapid clearance (CL = 75 ml/min/kg) and poor oral bioavailability (5%) in rats. The high clearance of PF-429242 was not predicted in vitro by human microsomes. Based on pharmacokinetic modeling, it was determined that administration of PF-429242 intraperitoneally every 6 h for 24 h at a dose of 30 mg/kg/dose would allow sufficient exposure to assess acute (24 h) in vivo efficacy.

The antilipogenic activity of PF-429242 was then evaluated in male CD1 mice ($n = 5$ per group) at doses of 10 and 30 mg/kg/dose i.p. every 6 h for 24 h. This resulted in greater than 80% reductions of HMG-CoA synthase gene expression[11] at both doses, dose related reductions in fatty acid synthase gene expression[11] (50% and 75%, respectively), and inhibition of the cholesterol and fatty acid synthetic pathways[12] (50% inhibition of both pathways at 10 mg/kg/dose; 80% for both pathways at 30 mg/kg/dose). LDL receptor gene expression[11] was not substantially altered at 10 mg/kg/dose but was 35% reduced at 30 mg/kg/dose, consistent with the smaller reduction of LDL receptor activity relative to reductions in cholesterol synthesis in cultured cells.

In summary, we have identified and characterized the biological efficacy of the first examples of site-1 protease inhibitors. Compounds with IC_{50}s of less than 10 nM were identified after 2 library iterations, an increase of greater than 200-fold relative to the initial high throughput screening hit. Characterization of the efficacy of PF-429242, a less potent compound with better physical properties, revealed that the S1P inhibitor prevented cleavage and nuclear translocation of the transcription factor SREBP, reduced expression of a variety of cholesterolgenic and fatty acid synthetic genes, and reduced cholesterol and fatty acid synthesis in cultured cells and in experimental animals. S1P inhibition is therefore an attractive therapeutic target for treating dislipidemia and a variety of cardiometabolic risk factors associated with altered cholesterol and fatty acid metabolism.

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8. The enzymatic activity and inhibition of S1P were measured fluorometrically using the MCA-conjugated peptidyl substrate, Ac-VFRSLK-MCA, essentially as described in Cheng, D.; Espenshade, P. J.; Slaughter, C. A.; Jaen, J. C.; Brown, M. S.; Goldstein, J. L. *J. Biol. Chem.* **1999**, *274,* 22805, with the following modifications: The reaction was conducted in 96-well plate format with an assay volume of 40 μL, the fluorogenic peptide substrate concentration was 20 μM, the purified His-tagged human S1P enzyme (secreted into serum-free pH 8.0 medium from stably transfected CHO-K1 cells and purified by nickel column affinity chromatography) concentration was 25 μg/mL (1 μg/well), the reaction was conducted for 4 h at 37 °C, compounds dissolved in DMSO were added such that the final DMSO concentration in the assay was 2.5%. Under these conditions, the assay exhibited a S/N ratio of 8 and a coefficient of variation of 15%. Confirmed S1P inhibitors did not exhibit fluorescence at either 360 or 460 nm nor did they quench fluorescence from a control well-containing MCA.
9. Enantiomers were separated and analyzed on preparative and analytical chiralpak AD columns, respectively, using a heptane/ethanol gradient with 0.2% DEA as mobile phase.
10. Proteolytic processing and nuclear translocation of SREBP were assessed as described by Cheng et al. (1999) op cit (Ref. 8).
11. HMG-CoA synthase, fatty acid synthase, and LDL receptor gene expression in cultured cells and in hepatic tissue samples was measured as outlined in Hawkins, J. L.; Gafvels, M.; Olin, M.; Lund, E. G.; Andersson, U.; Schuster, G.; Bjorkhem, I.; Russell, D. W.; Eggertsen, G. *J. Clin. Invest.* **2002**, *110*, 1191.
12. Cholesterol and fatty acid synthesis in cultured cells and in experimental animals were measured as outlined in Harwood, H. J., Jr.; Petras, S. F.; Shelly, L. D.; Zaccaro, L. M.; Perry, D. A.; Makowski, M. R.; Hargrove, D. M.; Martin, K. A.; Tracey, W. R.; Chapman, J. G.; Magee, W. P.; Dalvie, D. K.; Soliman, V. F.; Martin, W. H.; Mularski, C. J.; Eisenbeis, S. A. *J. Biol. Chem.* **2003**, *278*, 37099.
13. LDL receptor mediated LDL internalization was measured in cultured cells as outlined in: Harwood, H. J., Jr.; Pellarin, L. D. *Biochem. J* **1997**, *323*, 649, using DiI-LDL as the probe.