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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 633–637

Synthesis and biological activity of a novel squalene epoxidase inhibitor, FR194738

Masae Sawada,[a,*] Ken-ichi Washizuka[b] and Hiroyuki Okumura[b]

[a]*Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co. Ltd, 2-1-6 Kashima, Yodogawa-ku, Osaka 532-8514, Japan*
[b]*Medicinal Chemistry Research Laboratories, Fujisawa Pharmaceutical Co. Ltd, 2-1-6 Kashima, Yodogawa-ku, Osaka 532-8514, Japan*

Received 29 September 2003; accepted 18 November 2003

Abstract—The synthesis and biological properties of a novel squalene epoxidase inhibitor, FR194738, are described. This compound displayed potent in vitro inhibitory activities against squalene epoxidase and cholesterol synthesis, and lowered plasma cholesterol and triglyceride levels in dogs.

1. Introduction

Hypercholesterolemia is a major risk factor for the development of coronary heart disease (CHD).[1] Clinical trials have demonstrated that lowering plasma cholesterol levels by diet or drug intervention leads to benefits for hypercholesterolemic patients.[2] Although several inhibitors of HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, have been developed and used for this purpose,[3,4] they reduce non-sterol products such as farnesol, ubiquinone, dolichol and isoprenylated proteins,[5] some of which play important roles in the multivalent feedback inhibition of HMG-CoA reductase.[6] The resultant lack of feedback inhibition may lead to the induction of HMG-CoA reductase itself, and thereby attenuate the efficacy or even abolish the cholesterol lowering effects of HMG-CoA reductase inhibitors.[7,8]

Squalene epoxidase (SE) is a microsomal enzyme that catalyzes the conversion of squalene to 2,3-oxidosqualene. SE is located downstream in the cholesterol synthesis pathway, past the branch points for the biosynthesis of the non-sterol products mentioned above. Inhibition of SE should therefore have no effect on their production, but at the same time lead to cholesterol lowering with great efficacy. The allylamine class of antifungal compounds have been shown to be specific inhibitors of SE.[9] Depending on their precise chemical structure, allylamine derivatives can be highly selective for either fungal or mammalian SE. A number of efforts have been directed at the discovery of potent SE inhibitors for treatment of hypercholesterolemia. NB-598 was the first example of a potent and specific inhibitor of mammalian SE with an allylamine structure.[10] Other scaffolds have also been described.[11,12]

In the course of our research aimed at discovery of new SE inhibitors, we aimed to improve the lipophilic properties of NB-598 by modifying the thienyl–thienyl structure and to introduce polar substituents to increase polarity. As a result, we have discovered the novel SE inhibitor FR194738. In this paper, we describe the synthesis and biological properties of FR194738 (Fig. 1).

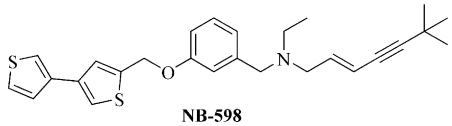

Figure 1.

* Corresponding author. Tel.: +81-6-6390-5482; fax: +81-6-6304-5367; e-mail: masae_sawada@po.fujisawa.co.jp

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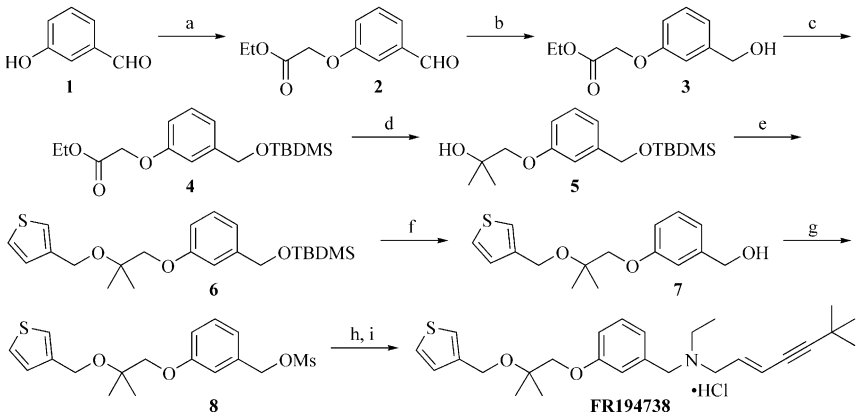
doi:10.1016/j.bmcl.2003.11.072



Scheme 1. (a) $BrCH_2CO_2Et$, KI, K_2CO_3, acetone, reflux, quant.; (b) $NaBH_4$, EtOH, 0 °C, 63%; (c) TBDMSCl, imidazole, DMF, 0 °C, quant.; (d) MeMgBr, THF, 0 °C, quant.; (e) (1) NaH, DMF, 0 °C to rt; (2) 3-bromomethylthiophene, 0 °C to rt, 67%; (f) TBAF, THF, 0 °C, 82%; (g) MsCl, Et_3N, THF, quant.; (h) (*E*)-*N*-ethyl-6,6-dimethyl-2-hepten-4-yn-1-amine, K_2CO_3, DMF, rt, 54%; (i) (1) HCl/AcOEt, rt; (2) recryst. from Hex/AcOEt, 63%.

2. Synthesis

The synthesis of our new SE inhibitor, FR194738, is outlined as shown in Scheme 1. Alkylation of *m*-hydroxybenzaldehyde with ethyl bromoacetate, followed by aldehyde reduction with sodium borohydride afforded the primary alcohol **3** in 63% yield over two steps. Protection of the alcohol as TBDMS, followed by reaction with excess methylmagnesium bromide afforded the tertiary alcohol **5** in quantitative yield. Alkylation of the alcohol moiety of **5** with 3-bromomethylthiophene in the presence of NaH–DMF, followed by deprotection of the silyl protecting group with TBAF in tetrahydrofuran afforded the alcohol **7** in good yield. Finally, activation of the alcohol as the mesylate (MsCl–Et_3N), followed by coupling with (*E*)-*N*-ethyl-6,6-dimethyl-2-hepten-4-yn-1-amine in the presence of potassium carbonate and conversion of the resulting free amine into the hydrochloride salt, afforded FR194738 as a white crystalline solid. The derivatives shown in Table 1 and 2 were prepared in a similar manner.

3. Biological methods

3.1. In vitro SE assay

Dog or rat liver microsomes and the homogenate of HepG2 cells, which are considered to be a suitable model for investigating lipid metabolism in human liver,[13] were solubilized with 2% Triton X-100. The assay mixture containing microsomes or cell homogenate, AMO-1680, FAD, NADPH, EDTA, [^{3}H]squalene and drug was incubated for 90 min at 37 °C. The reaction was terminated by the addition of ethanolic KOH. Non-saponifiable materials were extracted and separated on a silica gel TLC plate, and the radioactivity of 2,3-oxidosqualene was counted.

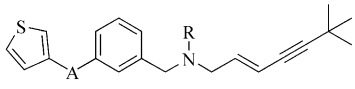
3.2. Cholesterol synthesis in HepG2 cells

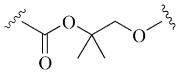
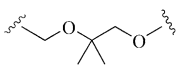
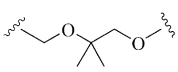
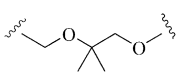
HepG2 cells were incubated overnight in a medium containing 10% human lipoprotein-deficient serum. The cells were preincubated for 1 h with drug and then [^{14}C]acetate was added. After 2 h of incubation, the cells were lysed and saponified with ethanolic KOH. Digitonin was added to the non-saponifiable lipids. Radioactivity of the digitonin coprecipitable sterols was counted.

3.3. Induction of HMG-CoA reductase activity in HepG2 cells

HepG2 cells were incubated for 18 h in a medium containing 10% human lipoprotein-deficient serum with or without drug. Thereafter, the cells were washed, scalped and suspended in buffer containing potassium phosphate, EDTA, KCl and Brij 97. After incubation for 15 min at 37 °C, the suspension was centrifuged at 12,000*g* for 15 min and the supernatant was used for measuring HMG-CoA reductase activity. HMG-CoA reductase

Table 1. Squalene epoxidase inhibitory activity of compounds with heteroatom-based linkers



Compd	A	R	Squalene epoxidase inhibitory activity IC_{50} (nM)		ClogP
			Human	Dog	
9		Et	23	370	7.48
10[a]		Et	5.4	34	7.30
11		Me	11	77	6.78
12		Pr	31	51	7.83
	NB-598		7.2	43	8.19

[a] Free form of FR194738.

activity was determined according to the method of Brown et al.[14]

3.4. Cholesterol-lowering activity

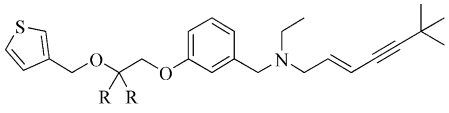
Male beagle dogs weighing 9–11 kg ($n=3$) were treated orally with FR194738 twice a day for 28 days. Blood samples were taken on the indicated days. On day 14, serum lipoprotein fractions were separated using an ultracentrifugation procedure. Plasma and lipoprotein lipid levels were determined enzymatically.

4. Structure–activity relationships

Table 1 outlines some of our results on the determination of SAR for our new series of compounds. NB-598 is a highly lipophilic compound (ClogP = 8.19). Since the highly lipophilic properties of compounds are related to poor solubility, permeation and absorption,[15] we attempted to increase polarity by incorporation of heteroatom-based linkers between the terminal thiophene, shown to be essential,[10] and the aryl amine portion of the molecule. The ester compound **9** was about 3 times weaker than NB-598 towards human SE, however, the ether **10** was slightly more potent (5.4 vs 7.2 nM). Of the *N*-alkyl analogues prepared, the *N*-methyl analogue **11** had the lowest lipophilicity, but the *N*-ethyl analogue **10** was most potent.

Variation of the geminal dimethyl moiety of **10** is shown in Table 2. It is clear from this table that only methyl is suitable at this position as other groups afforded much lower activity. Even a methylene group (compound **15**) was five times weaker against human SE. The hydrochloride salt (FR194738) of compound **10** was prepared and selected for further evaluation.

Table 2. Compounds with replacements for the geminal dimethyl moiety



Compd	R	Squalene epoxidase inhibitory activity IC_{50} (nM)		ClogP
		Human	Dog	
10[a]	Me	5.4	34	7.30
13	Et	120	ND	8.36
14	Allyl	160	ND	8.45
15	H	25	230	6.60
NB-598		7.2	43	8.19

ND, not determined
[a] Free form of FR194738.

Table 3. Squalene epoxidase inhibitory activity

Compd	Squalene epoxidase inhibitory activity IC_{50} (nM)			ClogP
	Human	Dog	Rat	
FR194738	9.8	14	25	7.30[a]
NB-598	7.2	43	36	8.19

[a] Value for free form.

5. Results and discussion

The novel derivative FR194738 was found to potently inhibit SE in HepG2 cell homogenate and liver microsomes in dogs and rats (Table 3), and the inhibitory activities were comparable to NB-598. The pharmacokinetic parameters in rats and dogs are shown in Tables 4 and 5. In rats, Cmax, $AUC_{0\text{-}8\ h}$ and bioavailability of FR194738 were higher than for NB-598. In dogs, NB-598 was undetectable in the plasma during the experiment. The superior pharmacokinetic properties of

Table 4. Pharmacokinetic parameters in rats

Compd	C_{max} (μg/mL)	T_{max} (h)	$AUC_{0\text{–}8\ h}$ (μg·h/mL)	BA (%)
FR194738	0.486	1.17	1.873	32.9
NB-598	0.329	1.67	1.392	16.5

Rats were given the compound orally, as a suspension in 0.5% methylcellulose at a dose of 10 mg/kg

Table 5. Plasma concentration in dogs

Compd	Dose (mg/kg)	Plasma concn (μg/mL)		
		1 h	4 h	24 h
FR194738	5	0.199	0.138	<0.05
	16	0.910	0.717	<0.05
NB-598	10	<0.05	<0.05	<0.05

Dogs were given the compound orally, as a suspension in 0.5% methylcellulose.

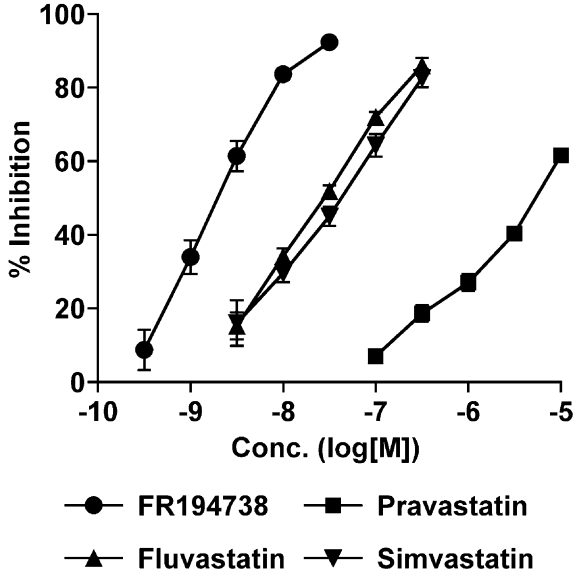


Figure 2. Inhibition of sterol biosynthesis in HepG2 cells by FR194738 and HMG-CoA reductase inhibitors.

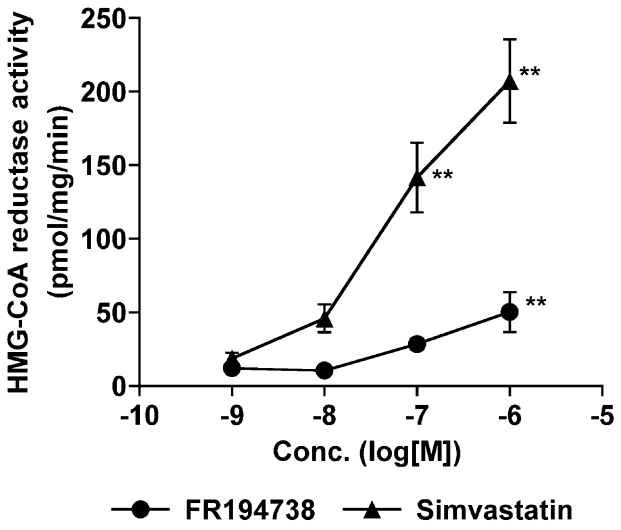


Figure 3. Induction of HMG-CoA reductase activity in HepG2 cells by FR194738 and simvastatin.

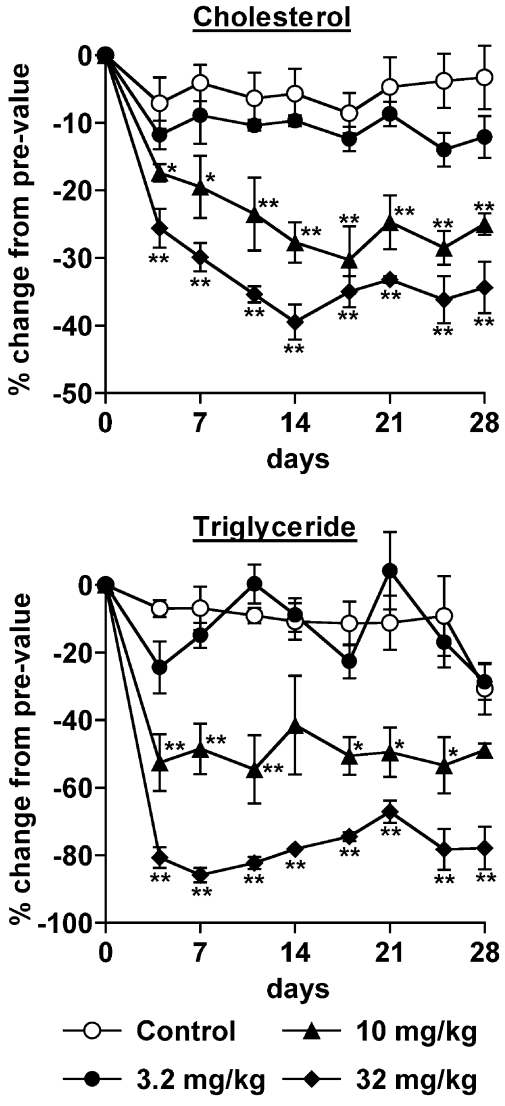


Figure 4. Lipid-lowering effects of FR194738 in dogs.

Table 6. Effects of FR194738 on plasma lipoprotein cholesterol levels and atherogenic index in dogs

Compd	Dose (mg/kg/day)	LDL cholesterol (% change)	HDL cholesterol (% change)	Atherogenic index (% change)
Control	—	+5	+1	+3
FR194738	3.2	−2	0	−2
	10	−47	−14	−38
	32	−61	−27	−47

Dogs were orally given the compound twice a day. On day 14, blood samples were taken and lipoprotein fractions were separated.
Atherogenic index = [LDL cholesterol]/[HDL cholesterol].
Figures represent % change from pre-value.

FR194738 compared with NB-598 may reflect the difference in lipophilicity and/or better metabolic stability.

The inhibitory effect of FR194738 in comparison to the clinically used HMG-CoA reductase inhibitors, simvastatin, fluvastatin and pravastatin, on cholesterol biosynthesis in HepG2 cells was next examined (Fig. 2). Among these compounds, FR194738 was the most potent, with an IC_{50} of 2.1 nM. The IC_{50}s of simvastatin, fluvastatin and pravastatin were 40, 28 and 5100 nM, respectively.

HMG-CoA reductase activity in HepG2 cells after long-term incubation with FR194738 or simvastatin is shown in Figure 3. Simvastatin caused a concentration-dependent increase of HMG-CoA reductase activity with a maximum increase of 18.8-fold. FR194738 also increased the enzyme activity but the degree of the change was less than simvastatin. The maximum increase by FR194738 was 4.6-fold. The difference between the two drugs could be attributed to the mechanism of action. HMG-CoA reductase activity is controlled through multivalent feedback regulation mediated by cholesterol and non-sterol metabolites of mevalonate.[6]

In vivo lipid lowering effects of FR194738 in dogs are shown in Figure 4. FR194738 dose-dependently lowered plasma cholesterol and triglyceride levels. The maximum decrease for plasma cholesterol levels was 40%, and that for plasma triglyceride levels was over 80%. Reduced secretion of VLDL, triglyceride-rich particles, by FR194738 might be responsible for the reduction in triglyceride levels in dogs. Khan reported that cholesterol is required for the secretion of VLDL.[16] In addition, FR194738 decreased LDL cholesterol levels more than HDL cholesterol levels, resulting in an improved atherogenic index (LDL/HDL ratio) (Table 6).

In summary, FR194738 possessed comparable in vitro potency to NB-598, but improved lipophilic and pharmacokinetic properties. FR194738 inhibited cholesterol biosynthesis in HepG2 cells more potently than the HMG-CoA reductase inhibitors, simvastatin, fluvastatin and pravastatin, with a minimal increase of HMG-CoA reductase activity. FR194738 lowered plasma cholesterol levels and improved atherogenic index in dogs, and has potential to be highly effective in the treatment of hypercholesterolemia.

Acknowledgements

The authors are grateful to Dr. David Barrett in the Medicinal Chemistry Research Laboratories for useful advice during the preparation of this manuscript.

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