Design, synthesis and evaluation of antihyperlipidemic agents combining antioxidant and antiinflammatory activity

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ABSTRACT: Atherosclerosis is a multifactorial disease in need of pharmaceutical treatment that can address a multiplicity of targets. We hereby describe the synthesis and evaluation of compounds in which antioxidant, anti-inflammatory as well as squalene synthase (SQS) inhibitory activities are combined by design, in a series of simple molecules, extending their potential activity against atherosclerosis. The activity of the synthesized antihyperlipidemic morpholine derivatives, in which we combined several pharmacophore moieties, was evaluated in vitro for antioxidant, inhibition of SQS and lipoxygenase activity as well as in vivo for antihyperlipidemic and anti-inflammatory effects. Most compounds had promising activity while structure-activity relationships are discussed. These results, taken together, show a promising alternative and novel approach for the design and development of antiatherosclerosis agents.

Key words: Aromatic tetrahydro-1,4-oxazines, synthesis, lipid peroxidation, triglycerides, total cholesterol, LDL-cholesterol, squalene synthase inhibitors, squalene synthase, lipoxygenase.

1. Introduction

Vaso-occlusive disease that results from atherosclerosis and thrombosis is considered the leading cause of death and morbidity in most parts of the world. Atherosclerosis is a progressive disease with several mechanisms participating in its manifestation and development. In various phases during disease development, hyperlipidemia, oxidative stress and inflammation play a major role.1,2

High levels of low-density lipoprotein (LDL) are recognized as the initiating event in atherogenesis. LDL can undergo extensive lipid peroxidation, resulting in the generation of modified LDL and the formation of atheromatic lesions.1 Lipoxygenases (LO), a family of enzymes mediating, among others, selective lipid oxidation, are suggested to participate in the development of atherosclerosis. Specifically, 12/15-LO in macrophages likely play an important role in continued oxidative modification of LDL by a variety of potential mechanisms. Hence, screening for 12/15-LO inhibitors as potential antiatherosclerotic agents is of interest, based on the evidence that these enzymes have proatherogenic properties.3

Compounds that inhibit cholesterol synthesis have proven to be useful in reducing levels of LDL and subsequent LDL oxidation in man, with beneficial effects against atherosclerosis. The main regulatory enzyme of the cholesterol synthesis pathway, 3-hydroxy-3-methylglutaryl-CoA reductase, can be inhibited by statins. However, these inhibitors interrupt the biosynthetic pathway early on and thus may...
prevent the formation of important compounds that are derived from the mevalonic acid pathway such as dolichols, ubiquinone and isoprenoid formation in general. Hence, the occurrence of undesirable side effects (i.e. hepatotoxicity and myotoxicity) arising from the inhibition of isoprenoid synthesis is a dose-limiting risk. As part of the efforts to discover novel hypocholesterolaemic drugs, agents that inhibit other steps of the cholesterol biosynthesis pathway have also been investigated. Squalene synthase (SQS), which catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate (FPP) to form squalene, is involved in the first committed step towards cholesterol biosynthesis. Inhibitors of this enzyme do not interfere with the biosynthesis of the above-mentioned isoprenoids, since this enzymatic step occurs after the branching point leading to isoprenoids. Furthermore, squalene synthase inhibitors lower not only plasma cholesterol, but also plasma triglycerides via a suggested FXR pathway. Several classes of squalene synthase inhibitors, such as quinuclidines or extended aromatic systems in order (i) to increase antioxidant activity, (ii) to increase lipophilic interactions with the binding site of SQS, and (iii) to provide some structural similarity with known non-steroidal anti-inflammatory drugs (NSAIDs) namely naproxen (and fenclorac hereby) in order to improve our understanding of structure activity relationships of this category of compounds.

2. Materials and method

2.1. Materials

All commercially available chemicals are of the appropriate purity and purchased from standard sources. [\(^3\)H] FPP (21.5 Ci/mmol), NADPH, FPP, and BSA were purchased from Sigma-Aldrich (Germany). Soybean Lipoxygenase (250 U/mL) was purchased from Sigma Chemical Co. (St Louis). For the in vivo experiments, Wistar male rats (200–250 g) were kept in a controlled temperature room (22±2 °C), having free access to laboratory chow and tap water, under a 12 h light/dark cycle.

2.2. Synthesis

General Procedure for the Preparation of the Final Compounds 1–15. The final products (figure 5) were obtained by the reaction of 2.2 mmol of 2-methyaminoethanol or 2-piperidinemethanol or trans-2-methylamino-cyclohexanol with 1.0 mmol of either 4-cyclohexyl-a-bromoacetophenone (compounds 1, 2, 3) or 2-bromoacetyl-6-methoxyanaphthalene (compounds 4, 5, 6) or 2-bromoacetylnaphthalene (compounds 7, 8, 9) or 2-Bromo-1-[4-(6-methoxy-2-naphthalene)-phenyl]-ethanone (compounds 10, 11, 12) or 2-Bromo-1-[4-(2-naphthalene)-phenyl]-ethanone (compounds 13, 14, 15), in anhydrous acetone (40 mL) at room temperature with stirring for 20–24 h. Acetone was then distilled off, ether was added to the residue, the mixture was washed with saturated sodium chloride solution, dried, and the products were isolated as hydrobromide salts. Identification and purity of all new intermediates and final compounds was established by means of melting point, infrared and \(^1\)H NMR (400 MHz) spectra, as well as elemental analyses. These data are available upon request.

2-(4-Cyclohexyl-phenyl)-4-methylmorpholin-2-ol hydrobromide (1), Yield 38%. 3-(4-Cyclohexyl-phenyl)-octahydro-1,4-pyrido[2,1-c]oxazin-3-ol hydrobromide (2), Yield 60%. 2-(4-Cyclohexyl-phenyl)-4-methyl-octahydro-1,4-benzoazoxin-2-ol hydro-bromide (3), Yield 78%. 2-(6-Methoxy-2-naphthalene)-4-methylmorpholin-2-ol hydrobromide (4), Yield 40%. 3-(6-Methoxy-2-naphthalene)-octahydro-1,4-pyrido[2,1-c]oxazin-3-ol hydrobromide (5), Yield 81%. 2-(6-methoxy-2-naphthalene)-4-methyl-octahydro-1,4-benzoazoxin-2-ol hydrobromide (6), Yield 70%. 2-(2-naphthalene)-4-methylmorpholin-2-ol hydrobromide (7), Yield 40%. 3-(2-naphthalene)-octahydro-1,4-pyrido[2,1-c]oxazin-3-ol hydrobromide (8), Yield 82%. 2-(2-naphthalene)-4-methyl-octahydro-1,4-benzoazin-2-ol hydrobromide (9), Yield 95%. 2-[4-(6-Methoxy-2-naphthalen)-phenyl]-
4-methylmorpholin-2-ol hydro-bromide (10), Yield 57%. 3-[4-(6-Methoxy-2-naphthalen)-phenyl]-octahydro-1,4-pyrido[2,1-c]oxazin-3-ol hydrobromide (11), Yield 67%. 2-[4-(6-Methoxy-2-naphthalen)-phenyl]-4-methyl-octahydro-1,4-benzoxazin-2-ol hydrobromide (12), Yield 60%. 2-[4-(2-naphthalen)-phenyl]-4-methylmorpholin-2-ol hydrobromide (13), Yield 40%. 3-[4-(2-naphthalen)-phenyl]-octahydro-1,4-pyrido[2,1-c]oxazin-3-ol hydrobromide (14), Yield 75%. 2-[4-(2-naphthalen)-phenyl]-4-methyl-octahydro-1,4-benzoxazin-2-ol hydrobromide (15), Yield 79%.

2.3. In vitro lipid peroxidation

Heat-inactivated hepatic microsomes from untreated rats were prepared as described.17,18 The incubation mixture contained heat-inactivated (90 °C for 90 sec) microsomal fraction, ascorbic acid (0.2 mM) in Tris-HCl/KCl buffer (50 mM/150 mM, pH 7.4), and the studied compounds (10 μM to 1 mM) dissolved in DMSO. The reaction was initiated by the addition of FeSO₄ (10 μM), and the mixture was incubated at 37 °C for 45 min. Lipid peroxidation of aliquots was assessed spectrophotometrically (535/600 nm) as 2-thiobarbituric acid reactive material.10

2.4. In vitro squalene synthase activity assay

SQS activity was evaluated by determining the amount of [³H] FPP converted to squalene as previously described.10,19 The assay was performed in 1 mL of 50 mM phosphate buffer, pH 7.4, containing 10 mM MgCl₂, 0.5 mM NADPH, rat liver microsomes (18 mg protein/mL), the test compounds dissolved in ethanol and [³H] FPP (0.5 mM, 0.27 Ci/mmol). The reaction was initiated by the addition of [³H] FPP and, after 10 min at 37 °C, was terminated with 1 mL 15 % KOH in ethanol. Following incubation for 30 min at 65 °C the mixture was extracted with petroleum ether. After washing with distilled water the upper organic phase was removed and counted with 3 mL scintillation liquid using a Beckman scintillation counter.

2.5. In vitro lipoxygenase activity assay

Lipoxygenase activity was determined using soybean lipoxygenase (250 U/mL) and sodium linoleate (100 μM) as substrate, in Tris/HCl buffer pH 9.0. The test compounds in 60% ethanol were added and the reaction was monitored for 6 min at 28 °C, recording absorbance at 234 nm.70

2.6. In vivo evaluation of antihypercholesterolemic and antihyperlipidemic activity

An aqueous solution of Triton WR 1339 was given ip to rats (200 mg/kg) and one hour later the test compounds (56 μmol/kg), dissolved in saline, or saline only were administered ip.21 After 24 h, blood was taken from the aorta and used for the determination of plasma total cholesterol (TC), LDL-cholesterol (LDL-C) and triglyceride (TG) levels, using commercially available kits.18

2.7. In vivo inhibition of carrageenan-induced rat-paw oedema

For the in vivo anti-inflammatory activity, Wistar male rats (150-180 g) were injected with 0.1 mL carrageenan (2% w/v solution in physiological saline) id into the right hind paw, the left paw serving as control. The test compounds (300 μmol/kg) were given ip after the carrageenan injection, and 3.5 h later the produced oedema was estimated as paw weight increase.

2.8 Protein determination

The protein content of microsomal fractions, used in lipid peroxidation and squalene synthase inhibitory assay, was determined according to Lowry’s method.23

2.9. Statistical analysis

Data are expressed as mean±standard deviation. Where indicated, statistical comparisons were made using Student’s t-test and a statistically significant difference was inferred if P<0.05.

3. Results and Discussion

3.1. Chemistry

The target compounds (1-15, figure 7) were synthesised in mostly good yields and via the spontaneous cyclisation of the corresponding hydroxyaminoketone intermediate to a hemiketal structure (figure 6). Structures and calculated24 lipophilicity values (CLogP) are presented in figure 5.

3.2. Antioxidant activity

The effect of the investigated derivatives on the non enzymatic peroxidation of hepatic microsomal membrane lipids after 45 min of incubation, expressed as IC₅₀ values, is shown in table 1. Under the same experimental conditions, 2,6-di-tert-butyl-4-methylphenol (BHT), a known potent antioxidant, and
probucol exhibited IC$_{50}$ values of 25 μM and >1 mM, respectively. The time course of lipid peroxidation, as affected by several concentrations of one of the most active compounds, 12, is depicted in figure 1. Most of the studied derivatives demonstrated significant antioxidant activity, stronger than that of the reference compound III (figure 7 and table 1). Thus, the performed structural modifications, primarily the extension of the conjugated system (compounds 10–15) led consistently to a considerable improvement of their antioxidant profile. As anticipated, and in accordance to previous observations, antioxidant activity seems to be favoured by conjugation or resonance effects of the further extended aromatic substituent (compare compounds 4–9 with 13–15), with an additional contribution to this by the positive resonance (+R) effect of the OMe substituent (i.e. compare 10–12 with 13–15). Further, lipophilicity, determined as CLog P values, in this series of derivatives seems also to correlate ($R^2$=0.70, $P<0.0001$) fairly well with antioxidant activity, in agreement with the notion that the lipophilic character of antioxidants contributes to the offered inhibition of lipid peroxidation, as it may facilitate their partitioning into biological membranes.

3.3. Inhibition of rat microsomal squalene synthase

Inhibition of the activity of squalene synthase, from rat liver microsomes, by the test compounds and expressed as IC$_{50}$ values, is shown in table 1. Most compounds inhibited squalene synthase activity significantly and dose-dependently as shown in figure 2 which depicts the activity of the most active compound 15. Bulky lipophilic substitution seems to improve the activity in this series of derivatives as exhibited for compounds 10–15, compared to the rest of the derivatives 1–9. This is in accordance with our previous studies, as well as studies on a series of 3-hydroxy-3-biaryl quinuclidines, that demonstrated an improved squalene synthase inhibitory activity for compounds with planar biaryl substitution while a directional requirement for the rigid biaryl side chain was identified. The biaryl substituent is considered to act as an isoster for isoprenyl subunits of the farnesyl chain of the endogenous enzyme substrate in cholesterol biosynthesis, and thus, an overall increase in lipophilicity seems to favor a better interaction between the molecule and enzyme, as expressed by lower IC$_{50}$ values.

<table>
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<tr>
<th>Cmpd</th>
<th>Inhibition of lipid peroxidation IC$_{50}$ (μM)</th>
<th>Microsomal SQS inhibition IC$_{50}$ (μM)</th>
<th>Lipoxigenase inhibition IC$_{50}$ (μM)</th>
<th>% Decrease compared to hyperlipidemic controls (56 μmol/Kg ip)</th>
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<tr>
<td>TC</td>
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<td>TG</td>
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<td>–</td>
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<tr>
<td>naproxen</td>
<td>n</td>
<td>–</td>
<td>–</td>
<td>24$^a$</td>
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</table>

$^a$$P <0.005$, $^b$$P <0.05$, $^c$$P <0.1$, $^d$not significant ($P >0.1$) (Student’s test), $^*data from Taraporewala et al$,$^*n.d.=not determined$
3.4. Antihyperlipidemic activity

The in vivo antihyperlipidemic activity of these derivatives is also demonstrated in Table 1. Experimental hyperlipidemia was successfully established 24 h after Triton WR 1339 administration, with an increase in plasma total cholesterol (TC), LDL-cholesterol (LDL-C) and triglyceride (TG) levels of 93%, 77% and 84% respectively, compared to normal values. All test compounds were found able to reduce the examined parameters in the plasma of hyperlipidemic rats (ca. 20–80% reduction), with the exception of two derivatives which had no effect on triglycerides (compound 11) or on LDL-cholesterol (compound 10). Under the same experimental conditions, probucol and simvastatin (used as antihyperlipidemic drugs), at the same dose, reduced plasma total cholesterol by 18 and 75%, LDL-C by 11 and 70%, and triglycerides by 18 and 0% respectively.

Compounds 2 and 12 were the most active in total cholesterol, LDL-C and TG reduction, while the activity of most of the other compounds was somewhat comparable to that of reference III (Table 1).

It seems that bulky, lipophilic substitution does not result in an overall increase in anti-dyslipidemic activity while other parameters, such as in vivo distribution and pharmacokinetics, may affect the in vivo order of activity of these compounds as compared to their in vitro activities.

3.5. Lipoxygenase inhibition

Lipoxygenases are important inflammatory mediators implicated in acute inflammatory reaction processes. Since cholesterol-loaded macrophages contain increased levels of 12-lipoxygenase, while both mRNA and protein for 15-lipoxygenase are present in atherosclerotic lesions, we investigated the effect of these derivatives on soybean lipoxygenase. Soybean lipoxygenase is inhibited by non-steroidal anti-inflammatory drugs in a similar manner to that of the rat mast cell lipoxygenase and is often used as a reliable means for evaluating lipoxygenase inhibitors.

Among the examined compounds, the methoxy-naphthyl derivatives 4–6 inhibited lipoxygenase activity in a concentration and time dependent manner (Table 1). Their IC50 values, after 6 min of incubation, were 20, 53 and 84 μM respectively, whereas the potency of the other compounds was much lower (IC50 values above 100 μM) (Table 1). The three active compounds 4–6 have incorporated in their structure a large structural moiety of naproxen (Figure 7), which is very likely to contribute to the above action. Naproxen's activity compared to
other anti-inflammatory agents is particularly high, inhibiting soybean lipoxygenase with an IC50 value of 24 μM.20 In contrast, no effect against lipoxygenase activity was noted for the respective naphthyl derivatives (7–9), confirming the positive contribution of the methoxy group in the activity of 4–6. Despite its potent in vivo anti-inflammatory activity,27,28 the non-steroidal anti-inflammatory agent fenclorac does not appear in the literature as a lipoxygenase inhibitor. Hence, the structural characteristics of the cyclohexylphenyl substituent of the derivatives 1–3 (that structurally partially simulates fenclorac) apparently does not favour lipoxygenase inhibitory activity.

3.6. In vivo anti-inflammatory activity

The anti-inflammatory activity of selected derivatives (2-6, 8, 9, 11-14) was evaluated by the method of carrageenan-induced paw edema which is a non specific inflammation maintained by the release of histamine and serotonin and later by prostaglandins.29 The examined compounds were administrated ip at a dose of 300 μmol/Kg right after the injection of carrageenan. The effect of compounds 2–6, 8, 9 and 11–14 and naproxen on paw edema is depicted in figure 4. Compound 3 demonstrated significant anti-inflammatory activity (63% edema inhibition) and higher than that of naproxen (51%).30 The activity of the other derivatives was also significant and comparable to that of naproxen at the same dose. The increased lipophilicity (figure 5) as well as the potent antioxidant activity of 3 may favor the antiinflammatory activity of this compound. Furthermore, the incorporated structural similarity of compounds 2 and 3 with the anti-inflammatory agent fenclorac, as well as that of compounds 4, 5, 6 with naproxen, may contribute to their increased antiinflammatory activity in vivo. The naphthyl derivatives 8 and 9 also exhibited good anti-inflammatory activity as expected, since several other naphthyl derivatives with antiinflammatory activity have been reported in the literature.31 Surprisingly, the activity of the "elongated" derivatives 11–14 was more or less retained as far as in vivo antiinflammatory action is concerned and comparable to compounds 5–8.

4. Conclusion

In summary, we designed, synthesized and evaluated a series of multiple acting antihyperlipidemic morpholine derivatives. The new derivatives combine several pharmacophore moieties in order to develop...
more active compounds aimed towards atherosclerosis. We evaluated in vitro (antioxidant, inhibition of SQS and lipoxygenase) and in vivo (anti-hyperlipidemic and anti-inflammatory) effects of these derivatives, demonstrating that they are more active than reference compounds. We further estimated structure-activity relationships that will be taken into consideration for the further design and development of new potentially more effective antiatherosclerosis agents.

References

Figure 6. Synthesis of the intermediate and final products 1-15. Reagents and conditions: (a) Pd(OAc)$_2$, Bu$_4$NBr, K$_2$CO$_3$, H$_2$O, Ar, 70 °C, 1.5-2 h; (b) Phenyltrimethylammonium tribromide, THF/H$_2$O, room temperature, 1.5 h; (c) i. acetone, room temperature, 20–24 h, ii. HBr/Et$_2$O, (d) NaOH, room temperature, 1.5 h, (e) CH$_3$NH$_2$, room temperature, 19 h.
Figure 7. Design of new (poly)aromatic antihyperlipidemic morpholine derivatives aiming to incorporate anti-inflammatory activity by combining structural moieties of known NSAIDs.

Submitted 21/07/2013
Accepted 16/09/2013