The liver X receptors (LXRα and LXRβ) are nuclear receptors that play a central role in the transcriptional control of lipid metabolism. LXRα and LXRβ are expressed in different cell types, by elevated intracellular levels of oxysterols deriving from cholesterol metabolism (e.g.: 24(S),25-epoxycholesterol). Synthetic LXR agonists promote cholesterol efflux, inhibit inflammation in vivo and inhibit the development of atherosclerosis in animal models. The ability of LXRs to integrate metabolic and inflammatory signalling makes them attractive targets for compounds potentially useful in human cardiovascular diseases (e.g. atherosclerosis). The present work is dedicated to the synthesis of three different classes of compounds, useful for the definition of the structural requirements for LXR activation, estimated from the cellular efflux of cholesterol from mouse peritoneal macrophages. SAR investigation have been performed by chemical modulations of known agonists, applying controlled modifications at their conformational, steric and electronic features.

1 Introduction

Coronary heart disease (CHD) is the leading cause of mortality in the western world, accounting for nearly 50% of all deaths [1]. Major risk factors for the development of CHD are hypercholesterolemia and dyslipoproteinemia. Many studies have identified decreased high-density lipoproteins (HDL) and increased low-density lipoprotein (LDL) cholesterol as major contributors to CHD. Consequently, many current therapies for the treatment of CHD, including statins, are aimed at lowering LDL or increasing HDL. Liver X receptors (LXRα and LXRβ or NR1H3 and NR1H2) belong to the type 2 family of the nuclear hormone receptor superfamily, working as transcription factors [2]. LXRα is expressed at high levels in liver, adipose tissue and macrophages, whereas LXRβ is ubiquitously expressed [3]. The receptors act as cholesterol sensors that are activated by elevated intracellular levels of oxysterols, deriving from cholesterol metabolism in different cell types [4]. LXRs form obligate heterodimers with retinoid X receptors (RXR) and regulate the expression of an array of genes involved in cholesterol homeostasis [5] and fatty acid metabolism [6]. One key LXR target gene is the ATP-binding cassette transporter A1 (ABCA1) which is involved in the process of reverse cholesterol transport (RCT) from macrophages to HDL in plasma [7]. Besides their function in lipid metabolism, LXRs have also been found to modulate immune and inflammatory responses in macrophages [8] and were recently reported as glucose sensors involved in liver carbohydrate metabolism [9,10]. Increasing RCT through LXR agonism is a potential therapeutic approach for a number of pathophysiological states including dyslipidemia, atherosclerosis and diabetes [11, 12]. To date, several distinct classes of agonists have been described in the literature [13–15] and patents [16], including natural ligands (e.g. 24(S),25-epoxycholesterol) and non-steroidal synthetic ligands (e.g. GW3965 [13] and T0901317 [17]). These ligands increase the expression of several genes involved in lipid metabolism and RCT including ABCA1, ABCG1, and ApoE. These compounds reduce, or even reverse, atherosclerotic processes in mouse models of atherosclerosis. Currently available synthetic LXR agonists, however, also activate triglyceride (TG) synthesis in the liver by the up regulation of SREBP-1c and FAS, which limits the utility of these LXR synthetic agonist.
2 Design

This research is focused on the synthesis of 3 different classes of compounds, in order to define the structural requirements for LXR ligands able to stimulate the cellular efflux of cholesterol from macrophages. SAR investigations have been performed by chemical modulations of known agonists, applying controlled modifications at their conformational, steric and electronic features. The first modification of known LXR agonist regarded GW3965: its propanolamine linker was replaced by a piperidine ring, which reproduced the chain conformation observed in the crystallized receptor-ligand complex, leading to compound 6 (type A in Figure 3). To avoid a charged quaternary nitrogen, compound 10 (type B) was also prepared, where the basic nitrogen had a topologically different position. As represented in Fig. 2, the rings are perfectly overlapped to the chain of GW3965 in the active conformation.

Compound 11 (type C) was obtained by replacement of the 3-CF₃-benziisoxazole moiety of the agonist F₃MethylAA [18] by a 3-OH-benziisoxazole. The binding mode of the agonist T0901317, co-crystallized with LXRβ (PDB code: 1PQC), had shown the importance of the interaction between the acidic carbinolic group and His435 for the activation of the LXR receptor [19]. Moreover, docking studies of the possible F₃MethylAA binding modes within the binding site of the GW3965/LXRβ crystal (PDB code: 1PQ6), supported the hypothesis that the CF₃ group could establish a polar interaction with His435. Maintaining the F₃MethylAA main scaffold, the 3-OH-benziisoxazole ring was introduced with the aim to have a polar group able to establish an hydrogen bond with His 435. This substitution was also carried out in order to characterize an original pharmacophoric moiety.

A small series of compounds (Table 2) were then synthesized as analogs of the potent LXR agonist class of maleimides [14]. The 5-benzylidene-hydaantoin class (Fig. 4) also includes terms endowed with EGFR Kinase inhibitory activity [20]. One of the (Z) isomer of this class (cmp. 13) fits the active conformation.
Fig. 3: General structures A, B and C obtained by modulating the synthetic agonist GW3965 and F3Methyl1AA.

Table 1: Synthesized analogs type A, B and C (6-12).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>type</th>
<th>R1</th>
<th>isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>A</td>
<td>OH</td>
<td>anty</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>OH</td>
<td>syn</td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>OCH₃</td>
<td>anty</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
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<td>10</td>
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<td>OH</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>OCH₃</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4: General structure of 5-benzylidene-hydantoin nucleus.

of GSK3987, the most potent compound of the maleimide class (Fig. 1). Docking studies of these 2 classes within the binding site of the GW3965/LXRα crystal (PDB code: 1PQ6), have shown different possible binding schemes for compound 13 and GSK3987 at the active site. Controlled modifications to the compound 13 were applied following the indications obtained by computational studies. In particular, the variations of substituents in positions N₁ and N₃ of the hydantoin nucleus and the double bound geometry were investigated in order to establish preliminary SARs for this series (Table 2).
Table 2: Synthesized hydantoin derivatives 13-23.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>(R_3)</th>
<th>isomer</th>
</tr>
</thead>
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<tr>
<td>13</td>
<td>Ph</td>
<td>PhCH(_2)</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>14</td>
<td>(n)-butyl</td>
<td>PhCH(_2)</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>15</td>
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</tr>
<tr>
<td>16</td>
<td>methyl</td>
<td>H</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>17</td>
<td>(n)-butyl</td>
<td>H</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>18</td>
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</tr>
<tr>
<td>20</td>
<td>Ph</td>
<td>H</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>21</td>
<td>Ph(CH(_2))(_2)</td>
<td>H</td>
<td>H</td>
<td>Z</td>
</tr>
<tr>
<td>22</td>
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</tr>
<tr>
<td>23</td>
<td>(n)-butyl</td>
<td>H</td>
<td>p-Cl</td>
<td>Z</td>
</tr>
</tbody>
</table>

3 Experimental

3.1 Chemistry

The compounds 6-9 were synthesized following the procedure described in scheme 1. Compounds 1 and 2 were submitted to Mitsunobu reaction and the following alkylation afforded compounds 8 and 9. The hydrolysis of methyl esters 8 and 9 with LiOH solution at r.t. provided compounds 6 and 7. Compound 10 was obtained starting from the methyl ester of isonipecotic acid, following the procedure described in scheme 2: alkylation of compound 3 with 3Br-(methylphenyl)-acetonitrile and hydrolysis of the nitrile group afforded 10 in good yield. Compounds 11 and 12 were synthesized in several steps as described in scheme 3. For compounds 13-23, a general and efficient four-steps procedure is described in scheme 4: starting from commercially available alkyl and arylalkyl primary amines, a small series of 5-benzylidene-hydantoins was synthesized.

![Scheme 1 Reagents and conditions](image)


3.2 Pharmacology

Cell culture. Mouse peritoneal macrophages (MPM) were cultured in RPMI with 10% FCS. Cells were incubated at 37°C, 5% CO\(_2\) seeded in 24-well plates and utilized at 80-90% confluence.
Incubation. MPM monolayers were washed with PBS and incubated for 24h in RPMI containing [1,2-\(^{3}H\)]cholesterol (4\(\mu\)i/ml) as described previously [21]. The labelling medium contained 1% FCS and 2\(\mu\)g/ml ACAT inhibitor to ensure that all labeled cholesterol was present as free cholesterol. Following 24h labelling period, cells were washed and then incubated in RPMI + 0.2% BSA with or without the indicated
compounds and 10µM of 9-cis retinoic acid (cRA) for 18h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propanol. These cells provide baseline (time 0) values for total [1,2-^3H]cholesterol content.

**Measurement of cholesterol efflux.** Stimulated and control monolayers containing [1,2-^3H]cholesterol were washed with PBS and incubated for efflux time (4h) in presence of cholesterol acceptor ApoA1 (10µg/ml). Cholesterol efflux was quantified by removing the cell medium and centrifuging it to remove floating cells. The radioactivity present in the incubation medium was determined by liquid scintillation counting and the percentage of radiolabeled cholesterol released (% efflux) was calculated as: (cmp in medium at 4h/ cmp at time 0) x 100.

### 4 Results and discussion

The variation of ^3H-Cholesterol efflux from mouse peritoneal macrophages was investigated for all the compounds described. A significant enhancement of the efflux was observed for the compounds 17 and 19 only (Fig. 9). The data obtained for the 5-benzylidene-hydantoin series allowed to formulate preliminary SARs for this class. Compound 13, which can be easily superposed to the maleimide ligand GSK3987 (not shown), was unable to stimulate cholesterol efflux, confirming the different binding modes suggested by computational studies. Compounds 17 and 19, carrying an aliphatic chain (n-butyl and n-hexyl) at position 1 and no substitution at position 3 of the hydantoin nucleus, showed significant efflux of cholesterol at both 10µM and 50 µM. Compound 18 (E isomer of compound 17) didn’t affect biological response, showing a possible geometrical selectivity for this class. Different substitutions at position 1 were also considered: introduction of methyl (16) or aromatic groups (20 and 21) gave complete loss of activity. These data suggested that in position 1 lipophilic substituents with no aromatic characteristics are required. Substitutions in position 3 were also investigated: the introduction of a methyl (14) or a benzyl (15) group didn’t afford active compounds. The importance of unsubstituted N3 could be explained with a possible hydrogen bond interaction between the receptor and the ligand. The introduction of meta-OH (22) and para-Cl (23) substituents at phenyl ring didn’t affect biological activity. Besides the ability in the induction of cholesterol efflux, compounds 17 and 19 have not shown cell toxicity at the active concentrations.

In summary, inclusion of the aliphatic chain linker of GW3965 into more rigid rings and replacement of 3CF3-benziisoxazole by 3OH-benziisoxazole for the agonist F3MethylAA gave derivatives unable to
induce significant efflux of cholesterol from primary peritoneal macrophages. The synthesis of a small 5-
benzylidene-hydantoin series led to compounds (17 and 19) able to induce a significant efflux of cholesterol
in cell cultures. Preliminary SARs were investigated for the 5-benzylidene-hydantoin derivatives, based on
the cholesterol efflux test on cultured macrophages.

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References

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Fig. 9: Efflux of cholesterol from primary peritoneal macrophages treated with T0901317 and compounds 17 and
19. Following the method previously described, the labeled monolayers were incubated with 10µg/ml of 9-cRA in
association or absence of compounds 17 and 19. The stimulated and control (C) cells were subsequently incubated with
ApoA1 10 µg/ml for 4h and the efflux was determined by liquid scintillation counting.


