A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action

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Abstract

A novel one-pot green chemical synthetic protocol involving the dual use of POCl3 and the catalysis of MWI has been developed for the preparation of the compound, 4-chloro-2-chloromethyl-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine. Further, it has been evaluated for antihyperlipidemic activity and found to possess it comparable to ezetimibe. Its docking study with six different molecular targets known to be implicated in hyperlipidemia, has revealed a good correlation between its high activity and very favourable docking interaction at one of these targets; NPC1L1.

Keywords: 2,4-dihalosubstitutedthieno[2,3-d]pyrimidine; MWI; Antihyperlipidemic; Ezetimibe; Molecular Targets, Docking

Introduction

Pyrimidine and its condensed derivatives show immense biological and medicinal significance and are considered as the most important and interesting heterocyclic ring systems [1]. Synthesis and antihyperlipidemic activity of various 2-substitutedthieno[2,3-d]pyrimidin-4(3H)-one derivatives have been reported [2-5]. One of such compounds, 2-chloromethyl-5,6,7,8-tetrahydrobenzo(b)thieno[2,3-d]pyrimidin-4(3H)-one (LM-1554) (Figure 1) was found to be promising as it possessed good antihyperlipidemic activity [5-7] and therefore, was subjected to detailed pharmacokinetic investigation [8]. The results of the studies revealed it to be poorly absorbed through the gastrointestinal tract (GIT). Though it was found to be active orally, it was inactive when administered by parenteral route. This indicated its probable site of action to be at the surface of the GIT (similar to the bile acid sequestering agents) [8].

Figure 1: Structure of LM-1554.
QSAR studies undertaken on its series [9], as well as, its analogous series [10] indicated the electronic parameter to be positively influencing the antihyperlipidemic activity of these compounds. Introduction of electron-withdrawing groups (EWG) at the 4-position of the pyrimidine ring in this series led to higher antihyperlipidemic activity. Thus, 4-chloro-2-chloromethylthienopyrimidines synthesised and evaluated exhibited much superior antihyperlipidemic activity than their 4-hydroxy counterparts [5,9]. Within this series of 4-chloro-2-substituted-methylthieno[2,3-d]pyrimidines; a compound 4-chloro-2-(chloromethyl)-5-(4-chlorophenyl)-thieno[2,3-d]pyrimidine (LM-1576) (Figure 2) was found to be much more active than LM-1554.

![Figure 2: Structure of LM-1576.](image)

The pharmacokinetic study of this potential lipid lowering compound, LM-1576; has been done and a rapid sensitive validated method for its quantification in rabbit serum using high-performance liquid chromatography (HPLC) has also been developed by us [11]. The validated method was successfully applied to a preclinical pharmacokinetic study of LM-1576 in rabbits. After oral administration of 100 mg/kg LM-1576 to rabbits, the main pharmacokinetic parameters $T_{\text{max}}$, $C_{\text{max}}$, $T_{\frac{1}{2}}$, $K_e$, $K_a$ and $AUC_{0-t}$ were 2 h, 1297.28 ng/ml−1, 0.495 h, 1.4 h−1, 3.99 h−1 and 2930.5 ng h−1L−1, respectively. Thus, this compound is indeed interesting and warrants a careful all round investigation.

Earlier, we have successfully synthesized a library of 2-(un)substitutedmethylthieno[2,3-d]pyrimidines in good yields and purity through the rapid one-pot condensation of appropriate thiophene o-aminoesters with aliphatic nitriles in presence of HCl and under MWI [12]. Further, we have also reported the novel one-pot synthesis of 2H-4-arylaminothieno[2,3-d]pyrimidines under MWI by making the dual use of formamide-POCl$_3$ combination as a reagent [13,14].

Earlier, we have reported an investigation into the mechanism of antihyperlipidemic action of a 2-substitutedthienopyrimidine; 2-chloromethyl-5,6,7,8-tetrahydrobenzo(β)thieno[2,3-d]pyrimidin-4(3H)-one (LM-1554) (Figure 1) through its molecular docking studies with six different molecular targets; Niemann Pick Cl Like1 protein (NPC1L1), ATP citrate lyase (ACL), C-reactive protein (CRP), lanosterol 14α-demethylase (LDM), squalene synthase (SqS) and farnesiod X-receptor (FXR) known to be implicated in the physiology of hyperlipidemia [15]. The interactions of LM-1554 were compared with those of their respective co-crystallized native ligands at their active sites. These comparisons were based on the docking parameters, as well as, types of interactions and vicinity with various amino acids in the active site pockets. Of these interactions of LM-1554 with NPC1L1 were found to be the most favourable.

Rationale

As LM-1576 has shown great potential for its development as a potent anti-hyperlipidemic entity, it was thought of interest to investigate its action at molecular levels, as well as, to develop a rapid facile method for its preparation and undertake the evaluation of its antihyperlipidemic activity and compare it with that of ezetimibe at dose levels closer to the drug.

Further, it was also thought of interest to develop a rapid one-pot synthetic protocol for LM-1576 by making dual use of POCl$_3$ as a solvent as well as, a chlorinating agent under the catalysis of MWI. Such a protocol was thought worth developing, as it could be adaptable to parallel synthesis of libraries of such compounds.
Though, the antihyperlipidemic evaluations of both LM-1554 and LM-1576 have been reported using preventive study and Triton WR 1339 models [5,9] and LM-1576 has been found to be superior in activity, in both these studies, the evaluations of both the compounds were at much higher dose levels. It was therefore thought worthwhile to evaluate both these compounds at much lower dose levels i.e. 10 mg/kg p.o., and compare their activity with that of the standard drug, ezetimibe (3 mg/kg p.o.). This could also give some insight to their mechanism of antihyperlipidemic action vis-à-vis that of this drug.

Graded dose levels of the standard as well as test compound were used to determine the ED_{50} and pED_{50} values (Table 1) which offer statistically best data set leading to significant QSAR.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose [mg/kg]</th>
<th>Total Cholesterol [mg/dl]</th>
<th>ED_{50}</th>
<th>pED_{50}</th>
<th>Triglyceride [mg/dl]</th>
<th>ED_{50}</th>
<th>pED_{50}</th>
<th>HDL [mg/dl]</th>
<th>ED_{50}</th>
<th>pED_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>-</td>
<td>62.53 ± 4.09</td>
<td>-</td>
<td>-</td>
<td>38.87 ± 2.46</td>
<td>-</td>
<td>-</td>
<td>35.37 ± 3.15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol-control group</td>
<td>-</td>
<td>169.4 ± 8.83*</td>
<td>-</td>
<td>-</td>
<td>107.6 ± 4.251*</td>
<td>-</td>
<td>-</td>
<td>37.14 ± 1.14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ezetimibe (Standard)</td>
<td>0.3</td>
<td>129.6 ± 8.18 [↑ 172]</td>
<td>2.6</td>
<td>1.585</td>
<td>100.2 ± 6.86 [↑ 683]</td>
<td>4.54</td>
<td>1.342</td>
<td>38.22 ± 2.23 [↑ 731]</td>
<td>5.09</td>
<td>1.293</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>101.2 ± 5.51***</td>
<td>1</td>
<td>0.40</td>
<td>75.54 ± 9.71 [↑ 10]</td>
<td>4.54</td>
<td>1.342</td>
<td>40.59 ± 3.44 [↑ 17.92]</td>
<td>43.13 ± 3.38 [↑ 31.5]</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>80.53 ± 5.18***</td>
<td>1</td>
<td>0.52</td>
<td>64.46 ± 5.38 [↑ 34.6]</td>
<td>4.54</td>
<td>1.342</td>
<td>43.13 ± 3.38 [↑ 31.5]</td>
<td>43.13 ± 3.38 [↑ 31.5]</td>
<td></td>
</tr>
<tr>
<td>LM-1554</td>
<td>25</td>
<td>143.7 ± 12.87 [↑ 15.05]</td>
<td>100.5</td>
<td>-0.002</td>
<td>87.10 ± 13.14 [↑ 19.37]</td>
<td>197.01</td>
<td>-0.294</td>
<td>38.06 ± 1.60 [↑ 1.78]</td>
<td>202.96</td>
<td>-0.307</td>
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<td>50</td>
<td>117.0 ± 3.57***</td>
<td>1</td>
<td>0.31</td>
<td>79.58 ± 7.26 [↑ 23.4]</td>
<td>4.54</td>
<td>1.342</td>
<td>40.96 ± 2.09 [↑ 6.77]</td>
<td>4.85 ± 2.14 [↑ 22.04]</td>
<td></td>
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<tr>
<td></td>
<td>100</td>
<td>89.49 ± 5.27***</td>
<td>1</td>
<td>0.48</td>
<td>72.28 ± 4.21 [↑ 32.7]</td>
<td>4.54</td>
<td>1.342</td>
<td>48.51 ± 2.14 [↑ 22.04]</td>
<td>43.36 ± 4.41 [↑ 12.66]</td>
<td></td>
</tr>
<tr>
<td>LM-1576</td>
<td>25</td>
<td>138.8 ± 9.92 [↑ 17.88]</td>
<td>93.11</td>
<td>0.031</td>
<td>103.3 ± 5.10 [↑ 1.40]</td>
<td>143.75</td>
<td>-0.157</td>
<td>37.84 ± 2.38 [↑ 4.41]</td>
<td>442.14</td>
<td>-0.645</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>107.5 ± 4.87***</td>
<td>1</td>
<td>0.36</td>
<td>91.00 ± 7.95 [↑ 15.3]</td>
<td>4.54</td>
<td>1.342</td>
<td>39.36 ± 1.19 [↑ 7.54]</td>
<td>43.36 ± 4.41 [↑ 12.66]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>82.29 ± 4.34***</td>
<td>1</td>
<td>0.51</td>
<td>72.10 ± 4.57 [↑ 32.9]</td>
<td>4.54</td>
<td>1.342</td>
<td>43.36 ± 4.41 [↑ 12.66]</td>
<td>43.36 ± 4.41 [↑ 12.66]</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Toxicity study for Ezetimibe, LM-1554 and LM-1576.
Each value represents the mean ± S.E.M. of six observation by ANOVA followed by Tukey’s test, *p < 0.001 statistically significance as compared to normal control group.

* P < 0.05 , ** P < 0.01, ***P < 0.001 statistical significance as compared to cholesterol fed diet group
Values in Parenthesis indicates, ↓% Reduction and ↑% Rise

Post study, the animals were kept for washing period for 15 days and the maximum survival time (MST) was observed to be 28 days for the control group. The MST observed for Ezetimibe, LM-1554 and LM-1576 treated groups was 35, 32 and 34 days, respectively, which were 7, 4 and 6 days more than the control group. Comparison in between MST of control group with LM-1554 and LM-1576 treated groups was found to be significant.

Citation: Kishor S Jain, et al. “A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action”. EC Pharmacology and Toxicology 7.2 (2019): 125-143.
Finally, the investigation into the mechanism of action of LM-1576, through its docking with various molecular targets, could also be explored, evaluated and compared with that done earlier for LM-1554 [15]. Niemann Pick C1 Like1 protein (NPC1L1) [16-20], ATP citrate lyase (ACL) [21-23], C-reactive protein (CRP) [24-26], Lanosterol 14α-demethylase (LDM) [27,28], squalene synthase (SqS) [29,30] and Farnesiod X-receptor (FXR) [31-33] are some of the attractive drug targets for the development of novel therapeutic agents for the treatment of hyperlipidemia. The X-ray crystal structures of these targets in complex with their native ligands were available with RSCB-Protein Data Bank (PDB). The evaluation and assessment of LM-1576, as a favourable ligand to these targets could be undertaken. Similar, study has been performed by us for the compound, LM-1554 [15].

Experimental

General

Melting points were determined using an electronic melting point apparatus (VeeGo-India; model MP-D) and are uncorrected. The purity of the synthesized compound was tested using precoated silica gel 60 F254 plates (Merck - India) and visualization was under UV light. The IR spectrum was recorded using KBr pellets (Perkin Elmer-USA; model Spectrum BX II FT-IR). The 1H NMR spectra were measured in CDCl3 using NMR spectrometer (Varian; model Mercury YH 300-MHz). Mass spectra were recorded on a GC-MS (Shimadzu-Japan; model QP2010). Microwave synthesizer (Questern Technologies Corp.- Canada; model-ProM) having monomode open vessel was used for the synthesis. Elemental analysis for LM-1576 was done using a Flash EA 1112 Thermofinnigan Instrument. The starting materials and reagents, 2-amino-3-carbethoxy-4-(4-chlorophenyl)thiophene [34], 1, chloroacetonitrile [35], 2, were prepared by reported methods, while, POCl3 was of procured commercially (Loba, India).

The total lipid profile was determined by using Infinite liquid cholesterol solution ready to use diagnostic kits (Accurex India Biomedicals, Mumbai, India). All the biological activity protocols carried out in this study were approved by the Institutional Animal Ethics Committee (SIPS/IAEC/2012-13/11) constituted under CPCSEA.

Procedure for preparation of 4-chloro-2-chloromethyl-5-(4-chlorophenyl)thieno[2,3-d]-pyrimidine (LM-1576)

A mixture of 2-amino-3-carbethoxy-4-(4-chlorophenyl)thiophene [34], 1, (0.01M, 2.82g), chloroacetonitrile [35], 2, (0.011 M, 0.83g) and 7.5ml of POCl3 (0.08M, 12.2g) was irradiated at 350W for 20 min in a microwave synthesizer. The progress of the reaction was monitored (TLC) at 5 minutes intervals. After completion of the reaction (20 minutes), the reaction mixture was allowed to cool to room temperature and poured into ice-water. The resulting precipitated solid was collected by filtration, washed with chilled water and dried. The crude product was recrystallized from methanol-chloroform mixture.

Yield: 75.25%; m.p. 62 - 64°C; IR (KBr): 3030 (C-H), 787 (C-Cl) cm⁻¹; 1H NMR (300 MHz, CDCl3) δ ppm: 1.65 - 1.70 (s, 1H, CH at 6), 4.90 (s, 2H, CH2Cl), 7.20-7.57 (m, 4H, Ar-H); EI MS m/z 329 (M⁺), 295, 293, 267, 230, 183, 174. Anal. C13H7Cl3N2S (329.63): C, 47.37; H, 2.14; N, 8.50. Found: C, 47.22; H, 2.10; N, 8.66.

Pharmacological activity

Healthy, unused Sprague Dawley rats (170 - 200g) of either sex were selected at random for the experiments. The animals were housed at a temperature of 30 ± 5°C and humidity of 40 - 50 ± 5% with 12h light and 12h dark cycles and were given food and water ad libitum, unless specified otherwise.

Preventive study for antihyperlipidemic activity evaluation (Cholesterol rich diet) [5,36]

Hyperlipidemia was induced in Sprague Dawley rats, by orally administrating a suspension of cholesterol (500 mg/kg body wt.) and cholic acid (250 mg/kg) in groundnut oil daily for seven days.
The animals were divided into five groups of six animals of either sex per group:

<table>
<thead>
<tr>
<th>Group I</th>
<th>The control group was administered daily with vehicle (2% acacia solution, as well as, normal saline) for seven days.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>The cholesterol-control group was administered daily with cholesterol suspension (conc. 500 mg/kg) and cholic acid (250 mg/kg) in groundnut oil as a single dose for seven days.</td>
</tr>
<tr>
<td>Group III</td>
<td>The standard group was administered daily with ezetimibe (3 mg/kg, p.o.) as suspension in 2% w/v acacia (aq.) for seven days in cholesterol fed animals.</td>
</tr>
<tr>
<td>Group IV</td>
<td>The test group-A was administered daily with LM-1554 (10 mg/kg, p.o.) as suspension in 2% w/v acacia (aq.) for seven days in cholesterol fed animals.</td>
</tr>
<tr>
<td>Group V</td>
<td>The test group-B was administered daily with LM-1576 (10 mg/kg, p.o.) as suspension in 2% w/v acacia (aq.) for seven days in cholesterol fed animals.</td>
</tr>
</tbody>
</table>

Blood samples were collected from the retro-orbital plexus of the eyes of the animals, initially and on the 8th day. The animals were kept on fasting 14h before the blood withdrawal. The samples were analyzed for the serum levels of cholesterol (total), triglyceride and HDL.

The entire animal study was of total seven days, wherein all animals survived and blood samples could be collected. Further, the animals were observed for 4 weeks post study for any deaths or ill effects. No untoward observations were recorded.

Docking Analysis

All the docking analyses of LM-1576 were performed using the Glide (Grid-Based Ligand Docking with Energetics) module incorporated in the Schrödinger molecular modeling package (Schrödinger, Inc., USA) running on an Intel Xeon based system with the Linux Enterprise OS. The Glide algorithm performs a grid-based ligand docking with energetics and searches for favourable positions, orientations and conformations of the ligand in the enzyme-binding pocket via a series of hierarchical filters. The shape and properties of the active site are represented on a grid by different sets of fields that provide progressively more accurate scoring of the ligand pose. These fields are generated prior to docking as a preprocessing step in the calculation and therefore need to be computed only once for each receptor. The binding site is defined by a rectangular box (Grid) confining the translations of the mass center of the ligand. The next step produces a set of initial ligand conformations through an exhaustive search of the torsional minima, and the conformers are clustered in a combinatorial fashion. Each cluster, characterized by a common conformation of the "core" and an exhaustive set of "rotamer group" conformations, is docked as a single object in the first stage. The search begins with a rough positioning and scoring phase that significantly narrows the search space and reduces the number of poses to be further considered to a few hundred over which computationally expensive energy and gradient evaluations will later be performed. In the following stage, the selected poses are minimized in the field of the receptor using a standard molecular mechanics energy function (in this case, the OPLS-AA force field) in conjunction with a distance-dependent dielectric model. The minimized poses generated by docking are then scored using the Glide Extra-Precision (XP) scoring function equipped with a variety of force field-based parameters accounting for solvation and repulsive interactions, lipophilic, hydrogen bonding interactions, metal-ligand interactions, as well as, contributions from Coulombic and van der Waals interaction energies, all incorporated in the empirical energy functions.

The starting coordinates of the protein structures - Niemann Pick C1 Like1 (NPC1L1) (PDB ID: 3QNT) [16], ATP-citrate lyase (ACL) (PDB ID: 3MWD) [21], C-reactive protein (CRP) (PDB ID: 1B09) [24], Human Lanosterol 14-Alpha-Demethylase (CYP51) (3LD6) [27], squalene synthase (SS) (PDB ID: 1EZF) [29] and farnesoid X receptor (FXR) (PDB ID: 1OSH) [31] were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) and further modified for Glide docking calculations.
Results and Discussion

Chemistry

POCl₃ was used as a solvent for cyclocondensation of 2-amino-3-carbethoxy-4-(4-chloro)phenylthiophene 1[34] and chloroacetonitrile 2 and also for the subsequent chlorination of the intermediate; 2-chloromethyl-5-(4-chloro)phenylthieno[2,3-d]pyrimidin-4(3H)-one 3 to give the target compound, LM-1576 in one-pot under MWI (Scheme 1).

Scheme 1: Synthetic pathway for the synthesis of LM-1576.

A solution of the starting materials, 2-amino-3-carbethoxy-4-(4-chloro)phenylthiophene 1[34] and chloroacetonitrile 2 in POCl₃ was irradiated LM-1576 under microwave irradiation (MWI). This protocol required only 20 min and afforded the product in good yields and purity (Scheme 1). The reaction proceeds through the formation of an imidoyl halide intermediate of the nitrile, which has enhanced electrophilicity to react with the o-amino ester, 1. The subsequent step involves the 4-hydroxy function with POCl₃ to form the corresponding condensed 4-chloroprimidine. The use of POCl₃ not only as a solvent or for the chlorination, but also as an acidic catalyst to form the imidoyl halide of chloroacetonitrile with a highly electropositive carbonyl carbon to readily undergo the cyclocondensation with the o-aminoester substrate under MWI appeared interesting and worth exploring. The reaction mechanism of this one pot protocol can be as under (Scheme 2). The target compound has been characterized by physical and spectral data.
Biological activity

Hyperlipidemia is a condition characterized by increased concentration of lipids (triglyceride, cholesterol) and lipoproteins (LDL and VLDL) in the blood. The antihyperlipidemic activity of LM-1576 was performed using cholesterol diet induced hyperlipidemic rat model (preventive study) and compared with our earlier reported compound LM-1554 and ezetimibe. The results of seven days preventive study model are given in table 2 (Figure 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Reduction in Total Cholesterol (mg/dl)</th>
<th>% Reduction in Total Triglyceride (mg/dl)</th>
<th>Change in Total HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM-1576</td>
<td>30.0 ± 3.30</td>
<td>25.3 ± 2.22</td>
<td>21.0 ± 3.11</td>
</tr>
<tr>
<td>LM-1554</td>
<td>29.1 ± 1.78</td>
<td>21.6 ± 2.20</td>
<td>20.5 ± 2.10</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>31.2 ± 1.10</td>
<td>24.4 ± 3.81</td>
<td>22.1 ± 1.62</td>
</tr>
</tbody>
</table>

*Table 2: Preventive study (lipid profile) data for LM-1576, LM-1554 and ezetimibe. Results are expressed as mean ± standard error, statistically significant (p < 0.05, t test, n = 6).*
During preventive study, oral administration of cholesterol rich diet comprising of cholesterol suspension (conc. 500 mg/kg) and cholic acid (250 mg/kg) in groundnut oil was administered to Sprague Dawley rats and blood samples were analyzed after seven days through retro-orbital puncture method. The effect on the lipid profile (cholesterol, triglycerides and HDL) was studied with an oral administration of LM-1554, LM-1576 and the standard, ezetimibe.

It was found that the LM-1576 at dose of 10 mg/kg p.o., showed significant changes in lipid profile of the test animals, i.e., decrease in total cholesterol, triglycerides and increase in HDL as compared with the control group.

LM-1576 effected reduction in % serum cholesterol levels of test animals, up to 30.0 ± 3.30%, more than that caused by LM-1554 (29.1 ± 1.78%) and comparable to that caused by ezetimibe (31.2 ± 1.10%) (Figure 3 and table 2). It also showed good % reduction in serum triglyceride levels of the test animals, up to 25.3 ± 2.22%, superior to that caused by LM-1554 (21.6 ± 2.20%) and slightly better than ezetimibe (24.4 ± 3.81%) (Figure 3 and table 2). Further, LM-1576 showed an increase in serum levels of % HDL up to 21.0 ± 3.11%, though, slightly lesser than ezetimibe (22.1 ± 1.62%) but, better than LM-1554 (20.5 ± 2.10%) (Figure 3 and table 2).

**Docking studies**

Niemann Pick C1 Like1 protein (NPC1L1), plays an important role in intestinal cholesterol absorption. Ezetimibe, first pharmacological inhibitor of cholesterol absorption has been shown to target NPC1L1 and thus, was used as a reference ligand in the present study. In case of the other five targets their native ligands were used for comparison. The results obtained from our molecular docking studies indicate that there is no significant difference in their binding mode with both the molecules occupying the similar co-ordinates of the active site. Also the glide score of the LM-1576 (-7.88) was observed to be higher than the ezetimibe (-6.31) indicating that LM-1576 is exhibiting anti-hyperlipidemic activity partially via inhibiting NPC1L1. The per residue interaction profile indicates that the molecule is anchored into the active site through balanced contribution of van der Waals (-34.93 Kcal/mol) and the electrostatic interactions (-34.9275) in the overall binding of LM-1576 to NPC1L1. A perusal of table 4a and figure 4a, indicates that the compound is stabilized into the active site through a series of favourable van der Waals interactions observed with Ile218 (-1.27 Kcal/mol), Leu216 (-1.95 Kcal/mol), Pro215 (-2.51 Kcal/mol), Thr128 (-3.48 Kcal/mol), Asn127 (-1.86 Kcal/mol), His124 (-3.843 Kcal/mol), Leu103 (-1.566 Kcal/mol), Gln206 (-0.825 Kcal/mol), Leu213 (-1.00 Kcal/mol), Pro215 (-1.10 Kcal/mol) and Ser53 (-1.236 Kcal/mol) residues while the high binding affinity is also attributed to equally significant electrostatic interactions observed with Asn127 (-0.284 Kcal/mol), His124 (-1.44 Kcal/mol), Ser102 (-2.433 Kcal/mol), Leu99 (-0.092 Kcal/mol), Ser98 (-0.071 Kcal/mol), Gln206 (-0.161 Kcal/mol), Leu213 (-0.153 Kcal/mol),

**Figure 3: Comparison of changes in lipid profiles caused by LM-1576, LM-1554 and ezetimibe in test animals during preventive study.**
Leu103 (-0.119 Kcal/mol), Pro215 (-0.116 Kcal/mol) and Ser53 (-0.294 Kcal/mol) residues. Furthermore, the compound was also seen to be engaged in a significant hydrogen bonding interaction with His124 and Ser102 residues which serve as an “anchor” guiding the 3D orientation of the ligand into the active site facilitating the steric and electrostatic interactions. Similar network of interactions were observed for LM1576 with other biological targets as well the details are summarized in tables 4b-4f and depicted in figure 4a-4f. The per-residue ligand interaction analysis suggests that the mechanical interlocking of these molecules is governed by the steric and electrostatic complementarity with the active site residues of the receptor. The docking scores of LM-1576 vis-à-vis LM-1554 and ezetimibe into the PDB structure of NPC1L1 point out better binding of LM-1576 at NPC1L1 (Table 3). Further, the perusal of figure 4a and table 4a also reveal better energies of interaction (Edocking, Evdw and Ecoul) with the molecular target (NPC1L1) for LM-1576 as compared to ezetimibe.

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<thead>
<tr>
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<th>Docking score w.r.t. target</th>
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<td>Co-crystallized ligand (Reference)</td>
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**Table 3:** Comparison of docking scores for LM-1554 [14], LM-1576 and co-crystallized native ligands into PDB structure of NPC1L1, ACL, CRP, LDM, SqS and FXR targets.

Such, binding scores and energies are not evident in case of LM-1576 with the other 5 molecular targets vis-à-vis their interactions with their respective ligands, viz., ATP citrate lyase (ACL), C-reactive protein (CRP), Lanosterol 14α-demethylase (LDM), squalene synthase (SqS) and Farnesoid X-receptor (FXR) (Refer figure 4b-4f and tables 4b-4f).

The figures 4a-4f depict the interactions of the amino acid residues in the active sites of the six molecular targets (listed above and under consideration for this docking study) with their respective native ligands as well as LM-1576. The tables 4a-4f detail the numerical data of these interactions like the docking scores, various energies of interactions and amino acid residue wise energies of interaction.

**Figure 4a:** 3D-docking of LM-1576 into PDB structure of NPC1L1

**Figure 4b:** 3D-docking of LM-1576 into PDB structure of ACL

Citation: Kishor S Jain., et al. "A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action". EC Pharmacology and Toxicology 7.2 (2019): 125-143.
A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action

Figure 4c: 3D-docking of LM-1576 into PDB structure of CRP (1B09).

Figure 4d: 3D-docking of LM-1576 into PDB structure of LDM (3LD6).

Figure 4e: 3D-docking of LM-1576 into PDB structure of SqS (1EZF).

Figure 4f: 3D-docking of LM-1576 into PDB structure of FXR.

Citation: Kishor S Jain, et al. “A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action”. EC Pharmacology and Toxicology 7.2 (2019): 125-143.
### Table 4a: Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target NPC1L1 (3QNT).

*a: All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.

*b: These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.

**: Ezetimibe was taken as reference native ligand for better comparison

d: Native ligand as co-crystallized in the PDB 3D structures.

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<th>Energy (kcal/mole)</th>
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### Table 4b: Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target ACL (3MWD).

*a: All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.

*b: These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.

**: Ezetimibe was taken as reference native ligand for better comparison

d: Native ligand as co-crystallized in the PDB 3D structures.

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### Table 4c: Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target CRP (1B09).

- **a**: All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.
- **b**: These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.
- **c**: Ezetimibe was taken as reference native ligand for better comparison.
- **d**: Native ligand as co-crystallized in the PDB 3D structures.
Table 4d: Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target LDM (3LD6).

a: All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.
b: These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.

c: Ezetimibe was taken as reference native ligand for better comparison
d: Native ligand as co-crystallized in the PDB 3D structures.
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**Table 4e:** Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target SqS (1EZF).

- **a:** All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.
- **b:** These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.
- **c:** Ezetimibe was taken as reference native ligand for better comparison
- **d:** Native ligand as co-crystallized in the PDB 3D structures.

**Citation:** Kishor S Jain, et al. “A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action”. *EC Pharmacology and Toxicology* 7.2 (2019): 125-143.
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Table 4f: Data for docking interactions (energies) of LM-1576 into PDB structure of molecular target FXR (1OSH).

<sup>a</sup> All amino acid residues were within 5Å from the ligand surface and 10Å from the centroid of the ligands.

<sup>b</sup> These amino acid residues though not visible in the figures, were actually on the rear side of this 3D pose and were observed in the interaction energy tables, as well as, in other poses.

<sup>c</sup> Ezetimibe was taken as reference native ligand for better comparison

<sup>d</sup> Native ligand as co-crystallized in the PDB 3D structures.
Conclusion

4-Chloro-2-chloromethyl-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine (1) has shown promising antihyperlipidemic activity in its preclinical evaluation and is found to be safe. Therefore, investigation into its pharmacodynamics was considered important. Molecular docking methods are now-a-days routinely used for predicting binding modes to proteins and energies of ligands. This technique was utilised for analysing the orientation of conformations and poses and assessing favourability of interactions of (1) into the binding pockets of six different molecular targets related to hyperlipidemia, mainly to gain some insights in its probable mechanism of action as an antihyperlipidemic entity. Based on the results (Figure 4a-4f, table 4) the compound seems to be acting through the inhibition of NPC1L1 (better docking scores and energy than, ezetimibe (Figure 5 and 6, Table 3 and 4), as well as, antihyperlipidemic activity comparable to it (Table 2)). It could also be acting through the inhibition of the other target, ACL (favorable docking energy as compared to its native ligand). The remaining four proteins could be ruled out as its likely targets. Thus, on this basis selective in vitro assays involving these two targets could now be the next step to confirm its mechanism of action.

Figure 5: Bar chart of docking scores for LM-1554, LM-1576 and co-crystallized native ligands for different antihyperlipidemic targets.

Figure 6: Bar chart of docking energies for LM-1554, LM-1576 and co-crystallized native ligands for different antihyperlipidemic targets.
Conflict of Interest
The authors have declared no conflicts of interest.

Supporting Data
The protein structures were prepared for Glide calculations by running the protein preparation wizard applying the OPLS-2005 force field. The crystallographic waters molecules were deleted and hydrogens were added to the structure corresponding to pH 7.0 considering the appropriate ionization states for both the acidic and basic amino acid residues. The most likely positions of hydroxyl and thiol hydrogen atoms, protonation states and tautomers of His residues, and Chi 'flip' assignments for Asn, Gln and His residues were selected using the protein assignment script. After assigning appropriate charge and protonation state, the prepared structures were further refined by subjecting to energy minimization until the average root mean square deviation (r.m.s.d.) reached 0.3Å.

The initial 3D structures of the co-crystallized ligands and LM-1576 were built using Maestro and then optimized by the LigPrep module (Schrödinger Suite) and the partial charges were ascribed using the OPLS2005 (Optimized Potentials for Liquid Simulations) force-field in the. This tool applies corrections to the structures (adjusts the bond lengths and bond angles), generates variations on the structures, eliminates unwanted structures, and optimizes the structures. The ligand partial charges were ascribed using the OPLS-2005 force-field and possible ionization states were assigned at a target pH of 7.0. The ligand geometries were optimized by subjecting to energy minimization using the LBFGS method until the gradient of 0.001 kcal/mol/Å was reached.

After ensuring that the protein and ligands were in the correct form, the receptor-grid was generated to define the active pocket for docking using the Receptor Grid Generation tool in Glide. All amino acids within 10Å of the co-crystallized ligand were included in the grid file generation except for Niemann Pick C1 Like1 (NPC1L1) (PDB ID: 3QNT) where the co-crystallized ligand is absent. In this case, the centroid of the residues Leu52, Thr106, Leu99, Ala101, Ser102, Ile105, Phe120, His124, Thr128, Glu201, Leu103, and Leu213 forming the active site of NPC1L1 was used to define the grid file. Default values were retained for the van der Waals scaling, and partial charges were assigned from the input structure, rather than from the force field, by selecting the use input partial charges option.

Following the grid generation, LM-1576 and the native ligands were docked into all the aforementioned targets with a view to identify how LM-1576 plays an important role in the control of hyperlipidemia. The extra-precision (XP) scoring function in Glide was used to rank the docking poses and to evaluate the binding affinity of the LM-1576 for the different targets. To analyze the mode of binding docked conformation with best glide (XP) score was selected.

Bibliography

Citation: Kishor S Jain., et al. “A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action”. EC Pharmacology and Toxicology 7.2 (2019): 125-143.


16. The starting coordinates of the protein structure - Niemann Pick C1 Like1 (NPC1L1) (PDB ID: 3QNT).


21. The starting coordinates of the protein structure - ATP-citrate lyase (ACL) (PDB ID: 3MWD).


24. The starting coordinates of the protein structure - C-reactive protein (CRP) (PDB ID: 1B09).


**Citation:** Kishor S Jain., *et al.* “A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action”. *EC Pharmacology and Toxicology* 7.2 (2019): 125-143.
A Novel 2,4-Dihalothieno[2,3-d]Pyrimidine as Antihyperlipidemic Agent: Synthesis, Biological Evaluation and Investigation into its Mechanism of Action


27. The starting coordinates of the protein structure - Human Lanosterol 14-Alpha-Demethylase (CYP51) (PDB ID: 3LD6).


29. The starting coordinates of the protein structure - squalene synthase (SS) (PDB ID: 1EZF).


31. The starting coordinates of the protein structure -farnesoid X receptor (FXR) (PDB ID: 1OSH).


