INTRODUCTION

Multiple Sclerosis (MS) is a chronic disorder affecting the Central Nervous System (CNS) through inflammation, demyelination, and neurodegeneration. Sphingosine-1-phosphate receptor (S1PR) modulators have been approved for the management of MS. Phosphorylated fingolimod mimics endogenous sphingosine-1-phosphate (S1P), a bioactive lipid that regulates remyelination and cell injury. Amiselimod was developed as a successor of fingolimod, with more specificity for S1PR1, and showed promising results until phase 2 clinical trials. This study utilized the fingolimod and amiselimod scaffolds, together with their critical binding interactions for the S1PR1 Ligand Binding Pocket, as templates for the in silico de novo design of high efficiency binding Lipinski rule-compliant molecules. A rigorous selection process identified two molecules, Molecules 003 and 019, deriving from fingolimod and amiselimod, respectively, which were deemed most suitable for further optimization.

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†Abbreviations: ALT, Alanine Aminotransferase; BBB, Blood-Brain Barrier; CNS, Central Nervous System; LBA, Ligand Binding Affinity; LBP, Ligand Binding Pocket; LLE, Lipophilic Ligand Efficiency; ML056, (3R)-3-amino-4-[[3-hexylphenyl]amino]-4-oxobutyl]phosphonic acid; MS, Multiple Sclerosis; PCA, Principal Component Analysis; S1P, Sphingosine-1-phosphate; S1PR1, Sphingosine-1-phosphate Receptor; TPSA, Topological Polar Surface Area; VS, Virtual Screening.

Keywords: Multiple Sclerosis, Sphingosine-1-phosphate Receptor 1, Fingolimod, Amiselimod, Protomol Generation, Drug Design

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achieved through a number of metabolic pathways. Firstly, the sphingosine kinase metabolizes the pro-drug to the active metabolite fingolimod-phosphate. Fingolimod-phosphate binds at minimal nanomolar concentrations to S1PR, located on lymphocytes and, due to its lipophilic character, readily crosses the blood-brain barrier (BBB) to bind to S1PR, located on neural cells in the CNS. By acting as a functional antagonist of S1PR on lymphocytes, fingolimod-phosphate blocks the capacity of lymphocytes to egress from lymph nodes, redistributing lymphocytes in the process. Fingolimod has an oral bioavailability of 93 percent after absorption, with steady-state blood concentrations taking 1 to 2 months with a daily dose. It is highly protein-bound, with a higher distribution in red blood cells. Biotransformation to the active S-fingolimod-phosphate occurs whereby elimination takes place primarily by CYP4F2 catalysis and is eventually excreted mainly through urine [4].

This study was a first step in the design of more selective S1PR, modulators, using the fingolimod and
amiselimod scaffolds and their critical binding interactions with the S1PR, Ligand Binding Pockets (LBP) as templates (Figure 1). A de novo approach was initially adopted to design and optimize novel ligands for the S1PR, In the second part of the study, potential novel S1PR modulators were sought using a Virtual Screening (VS) approach. The identification of novel S1PR modulators could potentially yield better molecules with neuro-protective properties beneficial in MS, without the risk of the fingolimod-associated bradycardia [5].

MATERIALS AND METHODS

In the de novo design process, X-ray crystallographic deposition 3V2Y describing the holo selective antagonist mimic, ML056, bound to S1PR, was selected as a template [6]. Molecular modeling was carried out in Sybyl-X [7]. The apo S1PR,LBP was mapped using the POCKET module of LigBuilder v1.2 [8]. This delineated the 3D-space within which novel molecular growth could be sustained. SAR studies from the literature [2] and the generation of 2D topology maps using Poseview [9] guided the modeling of four (labeled A-D in Figure 2) and three seed structures (labeled A-C in Figure 3) from the fingolimod and amiselimod conformers identified as optimally binding through conformational analysis, respectively. These were strategically planted into the S1PR_LBP, and novel moieties were computationally attached using the GROW module of LigBuilder v1.2 [8] at loci considered non-critical for binding. The analysis of drug-likeness of each resultant ligand was assessed based on the dual concepts of Lipinski rule-of-five and in silico-calculated Ligand Binding Affinity (LBA) (pKd) using the scoring function, X-Score v1.3 [10]. pKd may be defined as the sum that describes the overall stability of the interactions that occur in a ligand-receptor complex. Drug-likeness analysis based on the Lipinski rule-of-five meant that compliant molecules satisfied the criterion of having a molecular weight of not more than 500, a LogP not greater than five, hydrogen bond donors amounting to five or less, and hydrogen bond acceptors amounting to 10 or less [11]. The notion of having Lipinski rule-compliant molecules relates to the molecules being able to diffuse passively through the BBB.

In the VS approach, the fingolimod and amiselimod conformers identified as optimally binding through conformational analysis, together with the highest ranking ligands identified through the de novo approach, were uploaded to the online database ViCi [12] hosted by the University of Hamburg, in order to identify ligands of similar Three-Dimensional structural orientation and outer electronic configuration. The obtained hits were fil-

| Parent Molecule | Name of Molecule | Family | Formula | MWT | logP | Binding Score (pKd) | Chemical Score | Donors | Acceptors |
|-----------------|------------------|--------|---------|-----|------|-------------------|----------------|--------|-----------|
| Fingolimod      | result_003.mol2  | <1>    | C26H47NO3 | 421 | 4.93 | 7.85              | -100           | 4      | 5         |
| Amiselimod      | result_019.mol2  | <3>    | C16H19O3F3 | 316 | 4.47 | 7.17              | -20            | 5      | 6         |

Table 1. The properties of the best generated ligands of the de novo drug design that are Lipinski compliant with highest binding score, generated from LigBuilder v1.2 [8].
RESULTS

In the de novo approach, planting of the modeled seed fragments into the S1PR₁-LBP and use of the GROW algorithm of LigBuilder v1.2 [8] resulted in the generation of a total of 630 and 149 Lipinski rule-compliant fingolimod- and amiselimod-derived molecules, respectively. These were grouped into 23 (fingolimod-derived) and 12 (amiselimod-derived) pharmacophorically similar families (Figure 4).

The LBA (pKₐ) for the de novo-generated molecules

| Parent Molecule | Ligand   | Acceptors | Donors | Log p  | Total score | Cscore | Predicted in silico LBA within cognate receptor |
|-----------------|----------|-----------|--------|--------|-------------|--------|------------------------------------------------|
| Fingolimod      | 166      | 7         | 1      | 3.9034 | 6.39        | 4      | pKd= 5.69  
|                 |          |           |        |        |             |        | Predicted binding energy = -7.76 kcal/mol      |
| Molecule 003    | 170      | 5         | 1      | 4.1663 | 4.55        | 3      | pKd=7.85  
|                 |          |           |        |        |             |        | Predicted binding energy = -9.61 kcal/mol      |
| Amiselimod      | 69       | 4         | 1      | 3.4976 | 6.83        | 4      | pKd=5.72  
|                 |          |           |        |        |             |        | Predicted binding energy = -7.80 kcal/mol      |
| Molecule 019    | 500      | 2         | 0      | 2.879  | 6.66        | 4      | pKd=7.17  
|                 |          |           |        |        |             |        | Predicted binding energy = -7.95 kcal/mol      |

Table 2. The properties of the best generated ligands of the in silico drug design that are Lipinski compliant with highest total score, generated from Sybyl-X [7] and predicted binding energy and pKd from X-Score v1.3 [10].

Figure 4. Image showing the Lipinski-compliant molecules derived from the 4 seeds of fingolimod, grouped in 23 pharmacophorically similar families (A) and from the 3 seeds of amiselimod, grouped in 12 pharmacophorically similar families (B). Image rendered in Chimera 1.10.1 (UCSF-Chimera, Petterson EF et al, 2004) [20].

tered for Lipinski compliance using Mona [13]. A proto-
mol (Figure 5A) describing the total area available for ligand binding at the core of the S1PR₁ was generated in Sybyl-X [7] and used as a docking platform for the Lipinski rule-compliant molecules deriving from the online molecular database search. The ligands were re-filtered now based on their total affinity scores, and adapted Lipinski rule compliance in which LogP ranges between 3.15 and 4.96 were considered as predictors of CNS penetration [14].
The de novo-designed molecules deemed optimal were Molecule 003 (Figure 1B) generated from seed D of fingolimod (Figure 1A), having a pKd value of 7.85, a LogP value of 4.93 and a molecular weight of 421 daltons; and Molecule 019 (Figure 1D) generated from seed C of amiselimod (Figure 1C), having a pKd value of 7.17, a LogP value of 4.47 and a molecular weight of 316 daltons. For the fingolimod-derived molecules, the pKd value ranged from 5.17 and 7.85, with a molecular weight ranging between 313 and 481 daltons. The range for LogP was between 3.15 and 4.99. The pKd for the de novo-generated molecules derived from amiselimod ranged from 5.11 and 7.17, while the molecular weight ranged between 301 and 455 daltons. LogP ranged between 3.04 and 4.96.

Figure 5. Diagram showing the protomol (shown in cyan) together with the calculated key sites of fingolimod and amiselimod (Figure 5A). The super-imposed key site files mapped on the bound co-ordinates of fingolimod (pink) and amiselimod (green) within the S1PR, receptor (Figure 5B). Image rendered in Chimera 1.10.1 (UCSF-Chimera, Petterson EF et al, 2004) [20].

Figure 6. The best generated Molecule 003 (A) and Molecule 019 (B) with hydrogen bond acceptors featured in green dashed lines, hydrogen bond donors featured in magenta dashed lines and unsatisfied bonds featured in green spheres. Image rendered in BIOVIA Discovery Studio (Biovia, 2016) [18].
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Rived Ligand Number 166, with a Total Score of 6.39; Molecule 003-derived Ligand Number 170, with a Total Score of 4.55; Amiselimod-derived Ligand Number 69, with a Total Score of 6.83; Molecule 019-derived Ligand Number 500, with a Total Score of 6.6.

**DISCUSSION**

The POCKET algorithm of LigBuilder® v1.2 [8] was used to generate fingolimod- and amiselimod-bound LBP maps of the S1PR1 (Figure 5B). These maps were color-coded according to polarity highlighting different loci rich in hydrogen bond donors, acceptors, and hydrophobic groups, respectively. Visualisation of the fingolimod- and amiselimod-circumscribed LBPs, revealed very similar LBP maps. It was, however, interesting to note a different binding modality between amiselimod and fingolimod, with fingolimod exhibiting a somewhat curved binding modality and amiselimod assuming a 90-degree orientation within the S1PR1_LBP.

The optimal fingolimod- and amiselimod-derived molecules were selected based on the combined notions of high LBA (pKd) and low binding energy (kcalmol⁻¹).

**Table 3.** Table with properties of fingolimod, Molecule 003, amiselimod and Molecule 019 as displayed in SeeSAR [21] and X-Score v1.3 [10].

| Molecule          | Estimated Affinity | LLE  | MWT  | pKd  | LogP | TPSA | BBB Category |
|-------------------|-------------------|------|------|------|------|------|--------------|
| Fingolimod        | pM range          | Moderate | 337  | 5.69 | 3.27 | 68.1 | Negative     |
| Molecule 003      | nM range          | Moderate | 423  | 7.04 | 4.20 | 88.3 | Negative     |
| Amiselimod        | μM range          | Poor   | 378  | 5.72 | 2.95 | 77.3 | Negative     |
| Molecule 019      | μM range          | Poor   | 360  | 5.83 | 3.96 | 49.7 | Positive     |

Figure 7. Bar graph portraying the calculated *in silico* binding affinities and binding energies of the top 3 generated molecules, of each seed (cyan in colour) compared to fingolimod (peach in colour).
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The selection of lower-energy conformations was based on the premise that molecular stability is conferred by the positive ratio between intramolecular attractive to repulsive forces, and that the surplus of attractive forces will lower the overall potential energy of the molecule.

Optimal molecule 003, Figure 1B, generated from Seed D of fingolimod, seems to form critical hydrogen bonds with Val194, Tyr29, Asn101, and Arg120. The branching of the ligand is further stabilized through hydrophobic interactions (Figure 6A). Arg120 is an additional bond forged, not previously observed in the topology map of fingolimod.

Optimal molecule 019, Figure 1C, generated from Seed C of amiselimod, has hydroxyl moieties that form critical hydrogen bonds with Ser105, Tyr29, Asn101, and with the terminal nitrogen of Arg120. A terminal hydroxyl moiety remains unoccupied (green sphere). The trifluoromethyl moiety binds extensively with Phe125, Leu272, Leu297, Leu195, and intramolecularly with the phenyl group (Figure 6B). Ser105, Tyr29, and Arg120 are additional bonds forged, not previously observed in the topology map of amiselimod.

The hydrogen bond-forming capabilities of Tyr29 and its role in stabilizing both molecules require further consideration. The importance of this ligand-stabilizing interaction must be evaluated through in silico mutation and subsequent molecular dynamic stimulations. Validation of the importance of this specific interaction could have significant impact on the future iterative optimization.

The structure of Molecule 003 (Figure 1B) which was identified through the de novo approach is markedly different from those of fingolimod and amiselimod. The most striking differences are at the level of the ring, which has been opened in Molecule 003, and also at the level of the side chain, which, in the case of Molecule 003, now branches out to fill the available space more completely than do the side chains of fingolimod and amiselimod. The clinical consequences of these novel interactions must be investigated, particularly from an adverse effect perspective.

As evidenced by Table 3, Molecule 003 has the highest LBA (pKd) of 7.04 followed by Molecule 019 (LBA (pKd)=) and by amiselimod and fingolimod (LBA (pKd)= 5.83 and 5.69 respectively). LogP was highest for the new molecules, with Molecule 003 having a LogP of 4.20 when compared to fingolimod, whose LogP was 3.27. Molecule 019 had a LogP value of 3.96, whilst amiselimod had a LogP value of 2.95. The Topological Polar Surface Area (TPSA) is highest for Molecule 003, 88.3 and amiselimod, 77.3. Lipophilic Ligand Efficiency (LLE) is moderate with both fingolimod and Molecule 003, whereas poor with amiselimod and Molecule 019. Molecule 019 is predicted to penetrate the blood-brain barrier.

The docking process concerns the prediction of ligand conformation and orientation within a targeted binding site [15]. The protomol generated in the context of the VS approach was innovative and different from the LBP maps generated using the POCKET module of LigBuilder® v1.2 [8]. This indicated the existence of additional binding sites, which could potentially be utilized in an
attempt to identify novel suitable ligand interactions that could potentially favor efficacy and reduce side effects. Alternative site binding could also lead to hitherto unrecognized receptor conformational changes, potentially also contributing to partial or full modulation of the target receptor.

This in silico study is valuable in having identified two molecules whose high efficiency binding and associated physicochemical parameters support the hypothesis of high selectivity and oral bioavailability. This hypothesis requires molecular dynamics, in vitro, and in vivo validation.

In this context, we are publishing the results of a static in silico study. While conformational analysis, which was used to identify the optimal conformations of fingolimod and amiselimod within the S1PR₁-LBP, allowed single bond and ring rotations within a static binding pocket, it is envisaged that the next step in this study will be the setting up of a comparative Molecular Dynamics simulation study, in which the most important motions of fingolimod and amiselimod and the S1PR₁ will be compared statistically with those of molecules 003 and 019 using Principal Component Analysis (PCA). This will allow a better understanding of the nature of the interactions of molecules 003 and 019 with the S1PR₁, and will allow for enhanced atomic explanation of in vitro assay results.

Optimization of S1PR₁ modulators is still required in order to minimize adverse effects. Selectivity remains key in reducing adverse effects, with the exact cause of bradycardia remaining unclear but being apparently S1PR subtype 1 related. Other agents have exhibited other adverse effects in the course of undergoing clinical trials. Ponesimod (selective S1PR₁) was associated (2 percent of participants) with bradycardia, and with 1.2 percent and 0.9 percent of participants experiencing first and second degree heart block, respectively. Other reported effects were dyspnea and peripheral edema related to respiratory effects, together with altered alanine aminotransferase (ALT). Participants taking Siponimod (selective for S1PR₁, and S1PR₂) experienced second degree block and bradycardia, with one case of mortality in a patient with a history of coronary artery disease being reported. A study on Siponimod (BAF312) carried out by Gergely et al. proposes that bradycardia may be species-dependent. Ozanimod (selective for S1PR₁) exhibited serious adverse effects, specifically optic neuritis, uterine cervical squamous metaplasia, and somatoform autonomic dysfunction [16]. Amiselimod (selective for S1PR₁) showed promising results, but was discontinued after a successful phase two trial. Its withdrawal was not adverse effect-related, but seems to have been related to alternative fund allocation in what was termed a competitive regulatory landscape [16].

CONCLUSION

This study is a report of an initial in silico study that, given the adverse effect profile of fingolimod, seeks to identify structurally diverse lead molecules with clinical efficacy that is similar to or better than that of fingolimod, but that do not elicit the documented fingolimod-associated bradycardia. This study consequently proposes that this structural diversity be explored in the quest to identify clinically useful molecules with a more acceptable side effect profile.

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