Discovery of novel *Schistosoma mansoni* phosphodiesterase 4A inhibitors as potential agents against schistosomiasis

RUNNING TITLE: PDEs: potential targets for schistosomiasis

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Abstract

Background: Due to the urgent need for effective drugs to treat schistosomiasis that act through a known molecular mechanism of action, we focused on a target-based approach with the aim to discover inhibitors of a cyclic nucleotide phosphodiesterase from *Schistosoma mansoni* (SmPDE4A).

Material & Methods: To discover new inhibitors of SmPDE4A, homology models of the enzyme structure were constructed based on known human and protozoan homologues. The best two models were selected for subsequent virtual screening of our in-house chemical library.

Results and Conclusions: 25 library compounds were selected for experimental confirmation as SmPDE4A inhibitors and after dose-response experiments, three top hits were identified. The results presented validate the virtual screening approach to identify new inhibitors for clinically relevant phosphodiesterases.

Graphical Abstract:

Keywords: schistosomiasis, phosphodiesterase, virtual screening, inhibitors

INTRODUCTION

Schistosomiasis is a neglected parasitic disease caused by blood trematodes of the genus *Schistosoma*. It is a serious health problem, endemic in approximately 70 countries of the tropics and subtropics, with at least 230 million people treated per year [1]. In 2013 the disease reached Europe with an outbreak of urogenital schistosomiasis in Corsica infecting more than 120 people [2,3]. The life cycle of schistosomes is complex, involving free-living and parasitic stages. Cercariae live freely in water, can penetrate healthy human skin upon which they transform into schistosomules. These juvenile forms enter the blood vessels and reach the hepatic portal vasculature where they become sexually mature. Paired adult worms migrate to the bladder or the intestine depending on the species, and the females begin egg deposition. The eggs are either retained in host tissues where they induce inflammation, or voided in the feces or urine and continue the life cycle. Free-living miracidia are liberated from the eggs on contact with fresh water and become able to infect aquatic snails. Two rounds of asexual multiplication later, infective cercariae are released into water. The mammalian host's immune response to those eggs trapped in tissues induces the clinical manifestations of schistosomiasis [4].

Currently, an effective vaccine is lacking [5] and the only available treatment is with praziquantel, thanks to its efficacy, safety and low cost [6], with no new therapeutic options in sight despite the threat of developing resistance [7]. To anticipate the therapeutic failure of praziquantel and also due to the lack of efficacy against immature parasites, the development of new drugs to control the disease is urgent [8].

In neglected disease research, phenotypic screening together with target-based approaches are commonly used for drug discovery [9]. Careful selection of the molecular targets is key for success in this field. In this sense, the genomic era has had a tremendous impact on drug discovery in general, and on schistosomiasis drug discovery in particular [10]. The availability of the genomes of the three main clinical

schistosome species (namely *S. japonicum*, *S. mansoni* and *S. haematobium*) and their functional analysis, has given new insights into the biology of these flatworms, and given new impetus to schistosomiasis control efforts, especially with regard to the development of vaccines or the discovery of new agents based on new drug targets [11]. The integration of these areas with different bioinformatic tools used in medicinal chemistry is essential for therapeutic innovation in the field [12,13]. Moreover, further advances in the discovery of novel antischistosomal agents by molecular modeling approaches depends not only on the identification and validation of new pharmacological targets but also on the availability of chemically diverse small-molecule tools for experimental target validation studies [14].

In this work, the search for new drugs to treat schistosomiasis was based on a target-based approach with the aim to discover inhibitors of a cyclic nucleotide phosphodiesterase from *S. mansoni* (SmPDE), which has been recently proposed as a potential anti-*Schistosoma* target [15]. Phosphodiesterase (PDE) inhibitors have similarly been proposed as antiparasitic agents for a range of protozoan infections such as sleeping sickness [16], leishmaniasis [17], Chagas disease [18], giardiasis [19] or apicomplexan infections [20] because of the role of increased levels of cAMP caused by inhibition of the corresponding PDE [21]. Less is known about the cyclic-nucleotide signalling in helminths although it was reported the characterization of key proteins from this pathway in schistosomes [22,23] and also the vital role of cAMP-dependent protein kinase A in adult *S. mansoni* motor activity [24]. Moreover, recent studies on the druggability of SmPDE4A [15] encouraged us to search new inhibitors of this enzyme. In order to do this, the first objective was to generate homology models of this PDE that were subsequently used to virtually screen our in-house chemical library (MBC library) [25]. A group of 25 compounds was prioritized and tested in a biochemical *in vitro* assay as potential SmPDE4A inhibitors.

MATERIALS & METHODS

Homology modeling. In order to produce the most accurate model for SmPDE4A, different structures were retrieved from the PDB [26], including parasite PDEs 4I15 [27], 2R8Q [28], 3V93 [29] from *T. brucei*, *L. major* and *T. cruzi* respectively and hPDE4D2 (3SL4) [30]. Afterwards, the sequential alignment of the selected *S. mansoni* sequence with the four templates was performed. The alignment results showed that the *T. cruzi* structure was inadequate for use as template; consequently, three further models for SmPDE4A were built using the three remaining PDEs templates. For model building the templates were selected and the alignments between the template and the target were used for generating the tridimensional structure for the target sequence using ProMod-II in the SWISS-MODEL workspace [31,32]. Once the models were built, the estimation of the protein model accuracy is required. For that purpose, model quality is assessed with different metrics, as follows. The local composite scoring function QMEAN (Qualitative Model Energy Analysis) allows to discriminate good from bad models assessing geometrical aspects of the protein structure using several statistical descriptors. After building the models, energetic and geometric quality assessment of the tridimensional models generated was performed. A widely used tool is ProSA [33], that analyzes 3D models of protein structures for potential errors; being able to recognize errors and calculating an overall quality score for the protein structure. The Z-score value of the models was obtained using this application that calculates the standard deviation between the native fold of the protein and an ensemble of misfolds in terms of energy. In order to check the stereochemical quality of a protein structure, PROCHECK [34] has been applied analyzing residue-by-residue geometry. The Ramachandran plot shows energetically allowed regions for the backbone dihedral angles of all the residues, validating the stereochemical quality of the model. Finally, the models were analyzed by the software Verify3D [35]. This tool tests the accuracy of the 3D protein model in terms of energy. After the quality assessment, molecular dynamics studies were carried out for the selected models.

Virtual screening. It was performed employing Glide module [36] include in the

Schrödinger software package using both homology models and our MBC library [25].

Ligand preparation. The preparation of the library and the 2D-to-3D conversion was carried out using the LigPrep tool [37], included in the Schrödinger software package. This tool allows the preparation of molecules following different steps such as the addition of hydrogen atoms, the calculation of the ionization state of the molecules at a pH range, generation of possible tautomers and low-energy ring conformations, and a final energy minimization using the OPLS-2005 force field [38,39].

In order to perform the studies, physiological pH conditions were used to prepare the molecules, all of them were desalted and in the last step the compounds were minimized as default. A total number of 975 small molecules from the MBC library [25] using LigPrep were prepared in this way.

Two virtual screenings were carried out in selected homology models; using our in-house chemical library and applying the same protocol. In Model 1, conserved catalytic Gln, adjacent to one of the potential gating residues of P-pocket was selected as centroid of the grid while gating residues of the P-pocket were selected as grid centroids in Model 2. In both cases, the grid size was 16 Å, big enough to include almost all the catalytic pocket. In the grid generation, a scaling factor of 1.0 in van der Waals radius scaling and a partial charge cutoff of 0.25 were used. As reference compounds for virtual screening studies, we have selected seven benzoxaborole derivatives and roflumilast reported as SmPDE4A inhibitors in the literature [15]. A standard precision mode was used in the virtual screening, the ligand sampling was flexible, epik state penalties were added and an energy window of 2.5 kcal/mol was used for ring sampling. In the energy minimization step, distance dependent dielectric constant was 4.0 with a maximum number of minimization steps of 100 000. In the clustering, poses were considered as duplicates and discarded if both: RMS deviation is less than 0.5 Å and maximum atomic displacement is less than 1.3 Å.

Molecular dynamics. Both, the apo models and holo-complexes with the two most active compounds were studied through molecular dynamics simulation. As a force

field, the Optimized Potentials for Liquid Simulations-2005 (OPLS2005) [39] was applied to the system in Desmond Molecular Dynamic System [40] with the objective of analyzing the stability of the models. The structures of the proteins were preprocessed and refined carrying out H-bond assignment and calculation of the protonation state of the residues at physiological pH with a final restraint minimization, using the Protein Preparation Wizard tool [41] implemented on Maestro [42]. When the ligand was used in the molecular dynamics simulations RESP charges were calculated using Jaguar [43], optimizing the geometry for the compound using the method HF6-311++.

A triclinical periodic box and a TIP3P water solvation model were used for the prepared systems. The system was then neutralized adding the necessary number of counter ions. A NPT ensemble class with Noose-Hover thermostat and Martyna-Tobias-Klein barostat method and no constraints were applied. Steepest descendent method was applied for energy minimization and Desmond default protocol to equilibrate the systems. MD simulations were carried out on these equilibrated and prepared systems for a simulation period of 20 and 30 ns, energy and trajectory frames were captured after every 1.2 ps and 4.8 ps respectively The Simulation Quality Analysis tools guide the analysis regarding the quality of the molecular dynamics simulations and specific events during the process (such as distances between residues, etc) were analyzed by the Simulation Event Analysis tool.

Compounds studied. All the compounds tested in this work have a purity $\geq 95\%$ by HPLC and are collected in the MBC chemical library [25]. Structure of selected top hits, NPD-01243 [44], NPD-0327 [44] and NPD-0336 [45] was confirmed according to previously described procedures.

Expression and purification of SmPDE4A. The 6xHis tagged SmPDE4A gene was PCR amplified from a Trypanosome codon-optimized template synthesised by BaseClear (Leiden, The Netherlands), designed using the Integrated DNA Technologies tool (<https://www.idtdna.com/CodonOpt>) with manual checking to remove unwanted restriction sites from the sequences (the sequence of the 6xHis SmPDE4A

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full-length is included in Figure S1 from the Supporting information). The amplicon was cloned into a pOPINF [46] vector (BamHI x SphI) and recombinant Bacmids were generated. Baculovirus particles were produced by transfecting *S. frugiperda* Sf9 cells with recombinant bacmids, which were then used to transfect Sf21 cells for protein production. Protein expression was performed using *S. frugiperda* Sf21 cells grown in EX-CELL® 420 SF media (Sigma) and cells harvested 48 hrs post infection. The 6xHis tagged proteins were purified using the same purification strategy as described by Blaazer *et al* [47]. A typical purification yielded about 5 mg SmPDE4A from a 1-liter cell culture (Figure S2 from the Supporting information). The protein was stored in 50 mM Tris HCl pH 8.0, 100 mM NaCl, 2 mM DTT, 5 mM MgCl₂ and 5% glycerol.

Phosphodiesterase activity. These assays were performed using the PDELight™ HTS cAMP phosphodiesterase Kit (Lonza, Walkersville, USA), at 25 °C in non-binding, low volume 384-well plates (Corning, Kennebunk, ME, USA), essentially as described [48]. SmPDE4A (0.6 mg/mL) activity measurements (cAMP concentration at 2xK_m (6 μM)) were made in 'stimulation buffer' (50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM EDTA, 0.05 mg/mL BSA, pH 7.5). Single concentration measurements were made at 10 μM inhibitor concentration (triplicate measurements/assay, n=2). Dose response curves were made in the range 100 μM – 10 pM (triplicate measurements/assay, n=3). Test compounds were diluted in DMSO (final concentration 1%) and 2.5 μL aliquots were transferred to the 384-well plate, 2.5 μL of the PDE in stimulation buffer was added and mixed, 5 μL cAMP (at 2xK_m) was added, followed by incubation for 20 min at 300 rpm in 96-well plate shaker at room temperature. The reaction is terminated by the addition of 5 μL Lonza Stop Buffer supplemented with 10 μM pan-PDE4-like inhibitor NPD-0001 [16,47,49]. Then 5 μL of Lonza Detection reagent (diluted to 80% with reaction buffer) is added and the reaction incubated for 10 min at 300 rpm. Luminescence is read with a Victor3 luminometer using a 0.1s/well program.

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RESULTS AND DISCUSSION

Homology model of SmPDE4A. As no crystal structure of any of the known SmPDEs was available at the beginning of this work, homology modeling studies were carried out with the aim of generating different SmPDE4A models and develop a confident three-dimensional structure using comparative modeling. The proteins selected for this comparison were human PDE4D2 (hPDE4D2), and PDEs from parasites whose crystal structure were available, *Leishmania major* PDEB1 (LmjPDEB1), *Trypanosoma brucei* PDEB1 (TbrPDEB1) and *Trypanosoma cruzi* PDEC (TcrPDEC).

The target protein sequence of SmPDE4A was retrieved from UniProt [50], designated ID G4VPI6_SCHMA (GeneDB genomic sequence Gene ID: Smp_134140, <http://www.genedb.org>), with a total of 626 amino acids in its full-length sequence. This SmPDE isoform was selected since it was the closest to hPDE4 and our choice was reinforced by the recent identification of SmPDE4A as a relevant target due to its role in parasite motility and degeneracy [15].

The first step in the generation of the SmPDE4A homology model was to carry out a sequence alignment of the catalytic PDE domain of SmPDE4A (G4VPI6) with the catalytic domains of hPDE4D2, LmjPDEB1, TbrPDEB1 and TcrPDEC (Figure 1) in order to prioritize the potential templates for the homology process. This alignment allowed us to further study the identity between the different proteins and to identify important features such as discrepancies in critical residues of the catalytic site. As a result of the multiple sequence alignment of the catalytic domains, hPDE4D2 was identified as the most similar PDE to SmPDE4A, with an amino acid identity between the two sequences of over 60%, while the percentage sequence identity with the selected protozoan sequences was only approximately 30%. Furthermore, the binding site is quite conserved among all the sequences, particularly key residues such as invariant Gln553 (according to the numbering in SmPDE4A) and metal binding residues such as His348, His384, Asp385 or Asp502 are maintained in all sequences (Figure 1).

[Here, figure 1]

Another important feature is related to the presence of a parasite-specific pocket (also named P-pocket) in protozoan PDEs, which can be considered as an expansion of the catalytic site and could be of great interest for the development of parasite-selective inhibitors [51]. This pocket was first described in LmjPDEB1 crystal structure (2R8Q) [28] and subsequently observed in TcrPDEC (3V93, 3V94) [29] and TbrPDEB1 (4I15) [27] structures, showing Met and Gly (in the case of Tbr and LmjPDEB1) or Gly and Ser (in TcrPDEC) as gating residues in the secondary structure. However, this pocket is inaccessible in human PDEs due to the presence of different gating residues that makes the distance shorter. Moreover, the P-pocket in TcrPDEC is displaced compared to the other protozoan structures due to the shorter length of the region between gating residues [27,29]. For this reason, TcrPDEC was discarded as a template for the homology modeling process. Regarding SmpPDE4A, it was observed that although a hypothetical P-pocket could be localized in the same area as *L. major* and *T. brucei* some key residues are not conserved in SmpPDE4A, for example the presence of a Ser rather than a Gly residue at the gatekeeper position 552 (Figure 1). In order not to rule out any hypothesis, three models of SmpPDE4A based on the alignment of the 3D structures of hPDE4D2, LmjPDEB1 and TbrPDEB1 were constructed. To validate the three models, an evaluation process and comparative analysis of the three novel 3D structures was followed using different evaluation parameters and quality assessment metrics, as summarized in Table S1 from the Supporting Information. Model 3, based on TbrPDEB1, was rejected after analyzing the different energetic and geometric parameters because the least well-fitting parameters based on Ramachandran analysis and ERRAT values among others. The other two models were subsequently selected to carry out virtual screening studies.

PDEs: potential targets for schistosomiasis

Three-dimensional comparison of Model 1 (template hPDE4D2) and Model 2 (template LmjPDEB1), revealed that both are structurally very similar in topological folding and in the correspondence of the helices, as indicated by the RMSD-value of 1.068 between both structures (Figure 2). Also, the characteristic hydrophobicity of the catalytic pocket of the PDEs is conserved and consists of an important cluster of both aromatic and hydrophobic residues (Tyr343, His344, His348, His384, Met457, Tyr513, Leu520, Phe524, Phe525, Met541 and Phe556). The key difference between both models lies in slight dissimilarities in distances between gating residues (Met541 and Ser552) of the potential P-pocket in the range of 2 Å. This results in Model 2 preserving the characteristic P-pocket, slightly smaller than in LmjPDEB1, while in Model 1 the subpocket is almost as inaccessible as it is in the hPDE4 structure. Full details regarding distances between gating residues in the P-pocket can be found in the Supporting Information (Table S2).

Since there was not enough data at the time to choose which of the two models (Model 1 and Model 2) was better to be used as a proper target in the identification of novel inhibitors by virtual screening, it was decided to perform the study employing both models and attempt to gain experimental evidence to determine their comparative validity.

[Here, figure 2]

Virtual screening. Models 1 and 2 were subsequently used to carry out virtual screening studies. As a source of new hits, our in-house chemical library called MBC, recently described as an efficient source of new hits was employed [25]. As control compounds, seven benzoxaborole derivatives recently described as SmPDE4A inhibitors [15] were used, and also the well-known hPDE4 inhibitor roflumilast due to its reported activity in SmPDE4A [15].

PDEs: potential targets for schistosomiasis

After performing the virtual screening protocol using both models, the reference compounds were located near the top of the ranking (Table S3). For Model 1, 4 out of 8 positive controls are ranked in the first 35 positions of the screening, with all 8 positive controls in the best 10% ranking of the total number of poses. For Model 2, 6 out of 8 reference compounds presented docking score values in the best 9% of the complete ranking poses and 7 out of 8 in the best 13%. These results allowed us to validate our screening protocol and to confidently select the top-ranked compounds from the MBC library to be tested *in vitro*, following visually inspection. Analysis of the scaffolds corresponding to compounds with the best docking poses revealed that they could be grouped in 5 main chemical classes, namely thiazole, thiadiazole, indolinone, quinazoline and thiourea derivatives (Table 1). Moreover, the binding site of all of them was located in the catalytic site, showing interaction with the conserved Gln553 and Phe556 and other aromatic residues (Figure 3).

[Here, table 1]

[Here, figure 3]

Activity and inhibitory assay. A total of 25 virtual screening hits belonging to the main chemical classes were selected to be evaluated as inhibitors against SmPDE4A full-length (Table1). Initially, all prioritized compounds were tested at a single concentration of 10 μ M as inhibitor of SmPDE4A-mediated breakdown of cAMP. To this end, the SmPDE4A protein was expressed in Sf9 insect cells and purified as described in the Material & Methods section. Breakdown of cAMP was measured using the LONZA PDELight® kit. The single point determination indicated that from the virtual screening hits, three compounds, NPD-01243, NPD-0327 and NPD-0336, were able to inhibit the activity of the enzyme over 85%, whereas two other hits, NPD-03220 and NPD-0334, presented a moderate activity as SmPDE4A inhibitors with an inhibitory activity of 40 –~50% (Figure 4A). All compounds showing more than 50% inhibition of

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SmpDE4A activity were subsequently tested at different concentrations in order to determine their potencies as SmpDE4A inhibitors. As can be seen in Figure 4B, all three virtual screening hits inhibit SmpDE4A activity in a concentration-dependent manner with NPD-01243 > NPD-0327 > NPD-0336 with pK_i values of 7.0 ± 0.1 , 5.8 ± 0.1 and 5.7 ± 0.1 , respectively ($n = 3$ for each compound). Remarkably, iminothiadiazole NPD-01243 and NPD-0327 were previously described as human PDE7A inhibitors [52]. This human PDE is cAMP specific and its inhibitors, including these ones, have no emetic effects as those associated with PDE4 inhibition [53].

[Here, figure 4]

Molecular dynamic simulation of the complexes. To gain more information of the possible binding site and interaction of the protein with two of the most active compounds identified in this work, NPD-01243 and NPD-0327, molecular dynamic studies were performed. Firstly, to probe the stability of both models, molecular dynamic simulations of the apo-structure were performed. The apo structure of Model 1 was stable and no remarkable changes were observed during the simulations. In Figure 5A the different superimposed frames shown represent the initial point of the simulation (after carrying out the minimization and the relaxation protocols), a frame in the middle stage and also a frame in the final steps of the molecular dynamics period. The stability and the movements of the protein during the simulation can also be shown in the Root Mean Square Fluctuation (RMSF) plot (Figure 5B). Most of the residues in the protein show an RMSF value below 1.5 Å. Furthermore, in the P-pocket region RMSF values are below 1 Å, showing the stability of this part of the protein, and that the P-pocket region remains closed during the simulation.

[Here, figure 5]

For Model 2, the same protocol was followed for the molecular dynamic studies and more significant changes than in Model 1 were observed (Figure 6A). After the minimization and relaxation protocols and before the starting of the simulation phase, a significative rearrangement was observed consisting of a distance reduction between both gating residues of the potential P-pocket that made the subpocket inaccessible for a potential ligand. Furthermore, during the simulation the inaccessibility of this subpocket was maintained.

As a result, RMSF values during the molecular dynamics studies of Model 2 were higher than RMSF values in Model 1 (Figure 6B). Most of the residues presented an RMSF value over 1 and the potential P-pocket region especially showed high values compared to those observed with Model 1. While in the first model an RMSF in the range of 0.6-0.8 Å was found in this region, in the second one these values increased to 1.2-1.8 Å.

[Here, figure 6]

From the molecular dynamic studies with the apo structures of both models, it was concluded that Model 1 is very stable and during the simulation the P-pocket remains inaccessible from the catalytic site. In contrast, in Model 2 a significant rearrangement involving the P-pocket region was observed in which the walls of the loop are approaching each other, making the subpocket inaccessible.

Regarding holo simulations, the most potent ligands found in our virtual screening, NPD-01243 and NPD-0327, were studied. With the aim of deciphering the binding mode of these iminothiadiazoles and identify any important differences between both models. The main interactions found during the simulation processes are detailed in figures S3 and S4 from the supporting information. Moreover, a superposition of both binding modes for both compounds during the simulation showed that the binding modes for the two models are very similar. The main interactions found are hydrophobic, mainly with aromatic residues through π - π interactions, both face to face

and face to edge. However, ligand NPD-0327 is situated in a deeper part of the catalytic pocket in Model 2, due to the rearrangement in the P-pocket region (Figure 7). The presence of different His residues, such as His344, His348 and His384 and also several Phe residues, such as Phe524 and Phe556, in the catalytic site make the pocket hydrophobic, complementing the structure of the ligands: NPD-0327 contains a naphthyl and a phenyl substituent in the iminothiadiazole core, and NPD-01243 presents two phenyl substituents and an additional pyridine moiety. Also, some water-mediated hydrogen bonds are found, depicted in blue in figures S3 and S4. However, these water-mediated H-bonds are not maintained during all the simulations.

From these studies, it is possible to conclude that the binding site in both models is essentially the same and the critical interactions are aromatic and hydrophobic interactions.

[Here, figure 7]

Comparison of the experimental and theoretical structures. While the experimental studies of this work were in progress, the crystal structure for SmaPDE4A was released (PDB code 6EZU). A comparison of the X-ray structure and the homology models was performed and RMS values calculated. A high degree of similarity was observed, mainly in the catalytic binding site. In fact, X-ray structure and Model 1 are almost identical presenting a RMS value of 0.449 in the full structure, while comparison with Model 2 presents a RMS value of 1.046. Superposition of the published X-ray structure and the homology models (Figure 8) allowed us to conclude that, as predicted by the molecular dynamic simulations, the P-pocket is inaccessible in SmaPDE4A.

[Here, figure 8]

CONCLUSIONS

In order to identify potential inhibitors of SmPDE4A, three homology models of this enzyme were built based on the sequence retrieved from UniProt and hPDE4D, LmjPDEB1 and TbrPDEB1 structures as templates. Due to superior energetic and geometric parameters, the models obtained with hPDE4D and LmjPDEB1 as templates were selected to be used during the virtual screening campaign. After the enzymatic evaluation of the top 25-ranked virtual screening hits, five compounds showed the ability to inhibit the enzyme experimentally. These results validate our approach in the search of inhibitors of SmPDE4A and open the way to the development of better inhibitors that could be further developed as antischistosomal agents. Furthermore, molecular dynamic studies of both models point to the fact that SmPDE4A has no P-pocket or the pocket is inaccessible. While this work was in progress, the crystal structure for SmPDE4A was released. A comparison of our predicted structures and the now-published X-ray structure 6EZX confirmed the conclusions here presented with regard to the lack of P-pocket in SmPDE4A.

FUTURE PERSPECTIVE

The combination of different molecular modeling studies has shown a high value for the identification of drug candidates able to inhibit the selected enzyme experimentally. Further experimental studies will determine if the inhibition of SmPDE4A is a good approach to develop drugs for schistosomiasis.

EXECUTIVE SUMMARY

- Working hypothesis: Based on previous reported studies, SmPDE4A could be an interesting target for the development of anti-*Schistosoma* drugs.
- A medicinal chemistry approach based on the use of different chemoinformatic tools has allowed the identification of new hits.
- Virtual screening hits were validated experimentally as enzymatic inhibitors.

Figure legends

Figure 1. Sequence alignment of the catalytic domains of the potential templates for modeling SmpDE4A. The potential P-pocket region is depicted in green and key residues in the catalytic site are represented in cyan. G4VPI6_SCHMA, *Schistosoma mansoni* PDE4A; PDE4D_HUMAN, human PDE4D; Q6S996_LEIMA, *Leishmania major* PDEB1; Q8WQX9_9TRYP, *Trypanosoma brucei* PDEB1; Q53I60_TRYCR, *Trypanosoma cruzi* PDEC.

Figure 2. Structural superposition of Models 1 and 2 built in this work and selected to carry out further studies. In the zoom, view of the P-pocket. Model 1 is depicted in green and Model 2 in purple.

Figure 3. Structural superposition of some of the best binding poses found in the virtual screening studies depicted in yellow. In the zoom, view key residues that interact with the best poses of the virtual screening hits are shown, such as Phe524, Gln553, Phe556. Model 1 is depicted in green and Model 2 in purple.

Figure 4. (A) Single point determination of the top 25 virtual screening hits as inhibitors of SmpDE4A. Compounds were tested at 10 μ M concentration and PDE inhibitory activity was determined using the LONZA PDELIGHT[®] kit. NPD-0001 [47], a promiscuous PDE4-type inhibitor is included as positive reference. (B) Concentration dependent inhibition of SmpDE4A activity by the selected virtual screening hits. Data are fitted following a simple sigmoidal inhibition curve.

Figure 5. Molecular dynamic studies in the apo structure of Model 1. (A) Frames at different stages of the simulation are superimposed. Initial pose at 0 ns after minimization stage is depicted in green, the structure at the middle point of the simulation (10 ns) is depicted in gray and a frame from the final stage (20 ns) is shown

in orange. (B) RMSF plot of all the residues in the molecular dynamics simulation in Model 1.

Figure 6. Molecular dynamic studies in the apo structure of Model 2. (A) Frames at different stages of the simulation are superimposed. Initial pose at 0 ns after minimization stage is depicted in purple, the structure at the middle point of the simulation (10 ns) is depicted in gray and a frame from the final stage (20 ns) is shown in orange. (B) RMSF plot of all the residues in the molecular dynamics simulation in Model 2.

Figure 7. Binding mode superposition for compound NPD-0327 (A) and NPD-01243 (B) in both models at the middle point of the MD simulation (15 ns). Details of the binding mode and key residues are shown. Model 1 is depicted in grey and Model 2 in white. Ligands in Model 1 are depicted in green and in Model 2 in purple

Figure 8. Superposition of the SmPDE4A crystal structure (6EZU) in orange and Model 1 depicted in green (A) and Model 2 depicted in purple (B).

Table legends

Table 1. 25 top-ranked compounds selected to be biologically evaluated and experimental activity against SmPDE4A full-length.

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PDEs: potential targets for schistosomiasis

NPD-0326		-7.636	2	100.9 ± 3.1
NPD-03390		-7.567	1	120.0 ± 4.2
NPD-0279		-7.550	1	99.2 ± 0.5
NPD-0327		-7.540	2	0.9 ± 0.0 ^b
NPD-03219		-7.482	1	68.6 ± 1.3
NPD-03221		-7.404	1	88.8 ± 8.0
NPD-0328		-7.397	2	105.2 ± 4.7

NPD-0329		-7.373	2	104.5 ± 0.5
NPD-03217		-7.362	1	75.4 ± 2.7
NPD-0332		-7.357	2	96.6 ± 3.3
NPD-03218		-7.346	1	75.6 ± 2.3
NPD-0333		-7.337	2	93.9 ± 3.9
NPD-03220		-7.261	1	60.4 ± 1.9
NPD-0330		-7.214	2	102.9 ± 0.2
NPD-0338		-7.210	2	82.5 ± 7.0
NPD-0331		-7.145	2	91.5 ± 4.0

NPD-0334		-7.080	2	50.8 ± 3.6
NPD-0323		-6.967	1 and 2	94.7 ± 0.8
NPD-0336	HCl	-6.543	2	16.0 ± 0.2 ^c

^a: pK_i (NPD-01243)= 7.0 ± 0.1

^b: pK_i (NPD-0327)= 5.8 ± 0.1

^c: pK_i (NPD-0336)= 5.7 ± 0.1

Table 1. 25 top-ranked compounds selected to be biologically evaluated and experimental activity against SmPDE4A full-length.

Comp.	Structure	Docking score	Model	% SmPDE4A activity @10 μ M
NPD-0324		-7.942	2	100.7 $\pm$ 0.6
NPD-03215		-7.965	1	76.6 $\pm$ 0.6
NPD-01243		-7.913	1	0.2 $\pm$ 0.0 ^a
NPD-0335		-7.842	1 and 2	90.6 $\pm$ 5.1
NPD-0325		-7.749	2	104.5 $\pm$ 2.2
NPD-03216		-7.656	1	84.5 $\pm$ 0.9

major PDEB1; Q8WQX9_9TRYP, *Trypanosoma brucei* PDEB1; Q53I60_TRYCR,
Trypanosoma cruzi PDEC.

Figure 2. Structural superposition of Models 1 and 2 built in this work and selected to carry out further studies. In the zoom, view of the P-pocket. Model 1 is depicted in green and Model 2 in purple.

Figure 3. Structural superposition of some of the best binding poses found in the virtual screening studies depicted in yellow. In the zoom, view key residues that interact with the best poses of the virtual screening hits are shown, such as Phe524, Gln553, Phe556. Model 1 is depicted in green and Model 2 in purple.

A

B

Figure 4. (A) Single point determination of the top 25 virtual screening hits as inhibitors of SmPDE4A. Compounds were tested at 10 μ M concentration and PDE inhibitory activity was determined using the LONZA PDELight[®] kit. NPD-0001 [39], a promiscuous PDE4-type inhibitor is included as positive reference. (B) Concentration dependent inhibition of SmPDE4A activity by the selected virtual screening hits. Data are fitted following a simple sigmoidal inhibition curve.

Figure 5. Molecular dynamic studies in the apo structure of Model 1. (A) Frames at different stages of the simulation are superimposed. Initial pose at 0 ns after minimization stage is depicted in green, the structure at the middle point of the simulation (10 ns) is depicted in gray and a frame from the final stage (20 ns) is shown in orange. (B) RMSF plot of all the residues in the molecular dynamics simulation in Model 1.

A **B**

Figure 6. Molecular dynamic studies in the apo structure of Model 2. (A) Frames at different stages of the simulation are superimposed. Initial pose at 0 ns after minimization stage is depicted in purple, the structure at the middle point of the simulation (10 ns) is depicted in gray and a frame from the final stage (20 ns) is shown in orange. (B) RMSF plot of all the residues in the molecular dynamics simulation in Model 2.

A

B

Figure 7. Binding mode superposition for compound NPD-0327 (A) and NPD-01243 (B) in both models at the middle point of the MD simulation (15 ns). Details of the binding mode and key residues are shown. Model 1 is depicted in grey and Model 2 in white. Ligands in Model 1 are depicted in green and in Model 2 in purple.

A

B

Figure 8. Superposition of the SmPDE4A crystal structure (6EZU) in orange and Model 1 depicted in green (A) and Model 2 depicted in purple (B).

Supporting information

**Discovery of novel *Schistosoma mansoni* phosphodiesterase 4A inhibitors as
potential agents against schistosomiasis**

Table S1. Homology modeling summary: evaluation parameters and quality assessment analysis.

	Template	Identity (SM)	RMS(M/T)	QMEAN 4	Ramachandran			Verify 3D		Z-score	Errat
					Favoured	Allowed	Outlier	Min	Max		
Model 1	3SL4 (hPDE4D2)	60.11%	0.12	-2.06	97.9%	1,8%	0.3%	0.06	0.66	-8.32	90.85%
Model 2	2R8Q (LmjPDEB1)	31.87%	2.03	-4.04	94.9%	3.7%	1.5%	0.1	0.59	-7.06	79.78%
Model 3	4I15 (TbrPDEC1)	33.85%	3.76	-8.92	94.7%	3.4%	1.9%	-0.07	0.63	-6.3	72.69%

Table S2. Distance between gating residues in the P-pocket in the crystals used as templates and the models.

Protein	dCa-Ca (Å)	dCa-O (OH) (Å)	dSD-Ca (Å)	dSD-O (OH) (Å)	dCa-O (OC) (Å)	dSD-O (OC) (Å)
2R8Q (LmjPDEB1)	Met874-Gly886 7.5	- -	Met874-Gly886 7.0	- -	Met874-Gly886 8.5	Met874-Gly886 6.7
Model1 SmPDE4A	Met541-Ser552 7.1	Met541-Ser552 4.9	Met541-Ser552 4.7	Met541-Ser552 3.8	Met541-Ser552 8.4	Met541-Ser552 5.0
3SL4 (hPDE4D2)	Met357-Ser368 7.0	Met357-Ser368 4.7	Met357-Ser368 4.7	Met357-Ser368 3.8	Met357-Ser368 7.9	Met357-Ser368 5.1

Protein	dCa-Ca (Å)	dCa-O (OH) (Å)	dSD-Ca (Å)	dSD-O (OH) (Å)	dCa-O(OC) (Å)	dSD-O (OC) (Å)
2R8Q (LmjPDEB1)	Met874-Gly886 7.5	- -	Met874-Gly886 7.0	- -	Met874-Gly886 8.5	Met874-Gly886 6.7
Model2 SmPDE4A	Met541-Ser552 7.5	Met541-Ser552 7.6	Met541-Ser552 6.9	Met541-Ser552 7.2	Met541-Ser552 8.5	Met541-Ser552 6.6
3SL4 (hPDE4D2)	Met357-Ser368 7.0	Met357-Ser368 4.7	Met357-Ser368 4.7	Met357-Ser368 3.8	Met357-Ser368 7.9	Met357-Ser368 5.1

Table S3. Docking score of the control compounds (benzoxaboroles **1-7** and roflumilast) as validation protocol, in both virtual screening studies.

Comp.	Structure	Docking score (Model 1)	Docking score (Model 2)
1		-7.661	-6.342
2		-7.752	-6.952
3		-6.713	-8.847
4		-6.065	-5.814
5		-7.574	-6.035
6		-6.301	-5.603
7		-6.544	-6.627
Roflumilast		-7.356	-4.917

Features List

Name	type	location
His Tag	misc_feature	7..24
SmPDE4A	misc_feature	64..2160
STOP codon	misc_feature	2161..2163

atggcaccatcaccaccatcacagcagcgggtctggaagttctgttcagggcccgggatccATGGAACCTCAG
 AACAGACAAAGTCATCTCTAGCAATGATACGATTTCTTCTAGCCAGGAGCCTCAAT
 TGAGTCCGCAGATACCCGCCGTGGTGAAACCGCGCAGGGGGTCAATTTTTGTTA
 GCGCGCTTGCGGCAGAGCAGCACGGTGCGTCGAGTCTTCCACAGATAATACAG
 GGTCAATGATTGTCCGCCTGGGTAGATCGAGCACGGGTGGCGCTACAGCAGCGA
 CTTGACATCCAGCAGTTCTACAAATGGGATCACAGCCGTTTTACCGGATTCCAG
 CGTAGAGGGTGTTATCGTAACTCCCTTTGCGCAGGTCTGGTATCCATGCAAAGG
 ATACGTAATGCCTTTATACGCCTGACAGCGGCGCAGGTTTCTAACAAATATAATAT
 AACTACTGTTTTTGA CTCTGTACCCACGGGGAACCTCACACCCGATTCTTCAGACT
 ACAAATAATCGCCAATGAGACCTTAGAGGAACTTGAATGGTGTTTAAAGCAGCTG
 GAGAACATTCAAACGAAACGTCCAGTTAGCGACATGGCCTTCAGCAAATTTAAGC
 GTCTTTTGAACAAGGAACTGAACAGCTTCGGTGAAGCCGACAAGAGTAGGCACCA
 GATTTCCGCATACATTTGCGAAACGTTTCTGGAGACCGAGAAGGACGTGGAGACT
 AACGAAGAGATTGATTCCATGCTGGAGCGCCGACGTTCTCCGGCCAGTCACACA
 ATAGTACATCTGGCCAGGATACAAATACGACATCGAAGCGGCAAGCAAGCGGAAC
 AGGAGACCAAAATGCAAATCCAAATACGACAAGGACACCCGACACATCAAGCTCC
 GTGTCTAGCTCGGTTATCAAACACGGATCGGTTCAACTGGATCAATGTCCACAG
 AATCTCGCAAGAGTGCACAATTGAATGATTCTAGTGGTTTGCTTACGACGAAACTG
 CCAAGCAGCTCCAAACTGACAAGCCAAATGTAGATGACGGGAACGGTCCATTCT
 TGCCGATTCATGGCGTGGAACCTCCCAACGATAACGAGCTTGAGGAGCGTTTTAG
 CTTTGCCTGGACGAGTGGGGGGTGGATATTTTCGAAATCGACAGGTTGAGCAAC
 GGACATGCTTTAACACAGTCGCTTATCGGATCTTTCAAAAACGCGATCTGTTGAA
 GACATTTTGTATTGATCCACACGTGTTCTGTACGTTACCTTCTCCGGGTAGAGTCCA
 CTTACCATGCGGATGTGCCGTATCATAATAGTATGCACGCTGCTGATGTCCTTCAG
 ACGGCACATTTTTTGTCTCAGGCCGAAGCATTAGATGACGTTTTTTCCGACTTAGA
 AATTCTCGCAGTTCTCTTTGCCGCAGCAATTCATGATGTGGACCACCCCGGAGTG
 ACGAACCAGTTTTTGATCAATACGGGACATGAGCTCGCACTGCAATACAATGATG
 CAAGCGTACTTGAAATCATCATCTTTACATGGCGTTCAAGATCCTCACGGAGAAG
 GATTGCGACATATTTGCCAATCTGGGCGGGAAAAAGCGGCAAACCTTGAGACGTA
 TGGTAATAGAGTTGGTTTTGGCGACCGATATGAGTAAACATATGTCACCTTTGGCC
 GACCTTCGTACTATGGTGGAGACGAAAAAAGTCAGTGGGAGCGGTATGCTTAACC
 TTGATAATTACGCCGACAGGATACAGATATTACAAAATATGATACACTGCGCGGAT
 CTGAGTAATCCTGCAAAGCCGTTGCGCTTGACCGAAAATGGACAGGTAGACTTA
 TAGAGGAATTTTTCCGTCAAGGAGACAAGGAGCGTGAACCTCAGTCTCGAAATCTC
 TCCGATGTGCGATCGAGAAAGTGTGGAGGTGGAAAAGTCGCAGGTGTCATTATA
 GATTCGTCTGTCATCCTTTATGGGAGACATGGTGCGACCTGGTGACCCCTGTG
 CACAACCTATTTTAGACACGCTGGAGGACAATCGGGACTGGTACGAGTGTACAT
 AAAGGAGAGCAAAATGAAGGTCACACAATTAGCAAGGCCGAAACTGGCTACGGC
 GGCGGAAGATGATGAGGAAATCTCTACAACCTCAGGGAACACGtaa

Figure S1. Sequence of the 6xHis SmPDE4A full-length.

Figure S2. Purified SmPDE4A full-length (Star indicates the isolated enzyme).

Figure S3. Protein-ligand interactions in Model 1 and Model 2 in complex with NPD-01243 (A-B) during the molecular dynamics simulation studies.

Figure S4. Protein-ligand interactions in Model 1 and Model 2 in complex with NPD-0327 (A-B) respectively, during the molecular dynamics simulation studies.