

## Inhibitors of *Leishmania mexicana* Phosphoglycerate Mutase Identified by Virtual Screening and Verified by Inhibition Studies

(Pengenalpastian Perencat Fosfogliserasat Mutase daripada *Leishmania mexicana* melalui Saringan Maya dan Pengesahannya menerusi Kajian Perencatan)

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### ABSTRACT

*Cofactor-independent phosphoglycerate mutase has been proposed as a therapeutic target for the treatment of trypanosomatid diseases. In this paper, we report the identification of compounds that could potentially be developed as selective inhibitors of cofactor-independent phosphoglycerate mutase from Leishmania mexicana (LmiPGAM). Virtual screening was used in this search, as well as compounds identified by high-throughput screening. A ligand-based virtual screen programme, ultra fast shape recognition with atom types (UFSRAT), was used to screen for compounds resembling the substrate/product, before a structure-based approach was applied using AutoDock 4 and AutoDock Vina in a consensus docking scheme. In this way eight selected compounds were identified. In addition, three compounds from the Library of Pharmacologically Active Compounds (LOPAC) were selected from the published results of high-throughput screening of this library. The inhibitory effects of these compounds were tested at a fixed concentration of 1 mM. The results showed that seven compounds inhibited LmiPGAM activity and of these, two compounds (one each from high-throughput and virtual screening) showed substantial inhibition (i.e. 14% and 49% remaining activity, respectively). Taken together, the findings from this study indicate that these compounds have potential as novel inhibitors that specifically target LmiPGAM.*

*Keywords: Cofactor-independent phosphoglycerate mutase; glycolysis; Leishmania mexicana; virtual screening analyses*

### ABSTRAK

*Fosfogliserasat mutase bebas-kofaktor telah dicadangkan sebagai sasaran terapeutik bagi penyakit yang disebabkan oleh tripanosomatida. Di sini kami melaporkan pengenalpastian sebatian yang berpotensi untuk dibangunkan sebagai perencat kepada fosfogliserasat mutase bebas-kofaktor daripada Leishmania mexicana (LmiPGAM). Saringan secara maya telah diaplikasikan dalam kajian ini, selain daripada beberapa jenis sebatian yang dikenalpasti melalui saringan berprosesan tinggi. Program saringan maya berasaskan ligan, Ultra Fast Shape Recognition with Atom Types (UFSRAT), telah digunakan untuk menyaring sebatian yang menyerupai substrat/produk, sebelum pendekatan berasaskan struktur digunakan menerusi program AutoDock 4 dan AutoDock Vina di dalam skim simulasi pengikatan ligan kepada reseptor (docking) yang konsensus. Melalui kaedah ini, lapan sebatian terpilih telah dikenal pasti. Selain daripada itu, tiga sebatian daripada Library of Pharmacologically Active Compounds (LOPAC) yang dikenal pasti melalui kaedah saringan berprosesan tinggi terhadap perpustakaan ini yang telah diterbitkan turut dipilih untuk analisis lanjutan. Kesan perencatan kesemua sebatian ini telah diuji pada kepekatan yang ditetapkan pada 1 mM. Hasil analisis ini telah menunjukkan bahawa tujuh sebatian merencat aktiviti LmiPGAM, dengan dua sebatian (masing-masing daripada saringan berprosesan tinggi dan maya) menunjukkan perencatan yang ketara (14% dan 49% baki aktiviti). Secara keseluruhannya, hasil daripada kajian ini menunjukkan bahawa sebatian ini berpotensi sebagai perencat novel yang spesifik kepada LmiPGAM.*

*Kata kunci: Analisis saringan maya; fosfogliserasat mutase bebas-kofaktor; glikolisis; Leishmania mexicana*

### INTRODUCTION

The family Trypanosomatidae includes various parasitic protists among which are species of the genus *Leishmania*. They are responsible for causing a range of cutaneous and visceral diseases such as ulcers, destructive lesions and kala azar, an infection of the liver and spleen that is usually fatal if untreated. *Leishmania* species are a continuous threat to the inhabitants of many parts of the world. According to the World Health Organisation (WHO-NTD 2015), 12 million people from 98 countries worldwide are currently

infected with these diseases. At present, anti-leishmanial treatment depends primarily on chemotherapy, with several drugs now available as treatment options. However, the limitations of these drugs (high cost, injections required, painful and long course of treatment and serious side effects and resistance) highlight the need for the discovery of more affordable, less toxic and orally-consumable drugs (Golgher et al. 2011).

The essential role of carbohydrate metabolism in the survival of trypanosomatids has been well elucidated,

indicating that the glycolytic and/or gluconeogenic pathways play important roles (Verlinde et al. 2001). The cofactor-independent phosphoglycerate mutase (iPGAM), in particular, is an appealing drug target, as it exhibits no sequence or structural similarity with the corresponding enzyme in humans (Chevalier et al. 2000; Nowicki et al. 2009). Moreover, the enzyme is an essential component of both the glycolytic and gluconeogenic pathways and in this regard is of particular relevance in the pathogenic amastigote form of *Leishmania* species where gluconeogenesis (but not glycolysis) is an essential pathway (Naderer et al. 2006). By contrast, glycolysis is an essential pathway in the pathogenic bloodstream form of the related parasite *Trypanosoma brucei*.

Phosphoglycerate mutases (PGAMs, EC 5.4.2.1) (reviewed by Fothergill-Gilmore & Michels 1993) catalyse the interconversion of 3-phosphoglycerate (3PGA) and 2-phosphoglycerate (2PGA) in both the glycolytic and gluconeogenic pathways. In humans and vertebrates, as well as yeasts and certain bacteria, the enzyme relies on 2,3-bisphosphoglycerate as a cofactor for the catalytic function (cofactor-dependent dPGAMs). Cofactor-independent iPGAMs, on the other hand, require divalent metal ions to support their activities and can be found in protists (including *L. mexicana* and *T. brucei*), plants, fungi, archaea, algae, some nematodes and bacteria. While the dPGAMs are usually dimers or tetramers, the iPGAMs (classified as members of the metal-dependent alkaline phosphatase superfamily) are predominantly monomeric enzymes. Since the two enzymes are evolutionarily unrelated, drug design against iPGAM from trypanosomatid parasites has been of particular interest. Amino acid sequence analysis of iPGAMs shows that they occur in two families, with the enzymes from trypanosomatids and plants in one family, and bacterial, algal and nematode enzymes in another family (Blackburn et al. 2014). The enzymes in the two families are very similar, but differ in the details of their dynamic properties and modes of action.

Crystal structures of *L. mexicana* iPGAM (*Lmi*PGAM) (PDB: 3IGY) (Nowicki et al. 2009), *T. brucei* iPGAM (PDB:3NVL) (*Tbi*PGAM) (Mercaldi et al. 2012), various *Bacillus* species (Jedrzejewski et al. 2000; Nukui et al. 2007) and *Staphylococcus aureus* (Roychowdhury et al. 2015) have been reported. All iPGAM structures show a two-domain monomeric enzyme, which may be trapped in both open apoenzyme and closed ligand-bound conformations (Figure 1). In its closed conformation, the active site was inaccessible to the solvent, a situation which may correlate with disappointing results from high-throughput inhibitor screening (Crowther et al. 2014).

Since the main goal of our research was to identify new drugs for the treatment of leishmaniasis, a series of virtual screening strategies has been carried out with the aim of finding novel drug-like inhibitors. A ligand-based drug design experiment was employed by initialising similar compounds that resemble the natural ligands occupying the catalytic site. Subsequently, a series of docking and scoring experiments were conducted following a structure-based

drug design approach, before compounds were selected for *in vitro* inhibition analysis. In parallel, a preliminary high-throughput screen was conducted at the National Institutes of Health (NIH), Bethesda, MD, USA (<http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=504639>), the goal of which was to assess the ability of specific compounds to inhibit enzyme activity *in vitro*. Both the virtual and high-throughput screening approaches offer new hope for the discovery of novel drug-like inhibitors that specifically target *Lmi*PGAM.

## MATERIALS AND METHODS

### PROTEIN EXPRESSION AND PURIFICATION

The bacterial expression of C-terminally His<sub>6</sub> tagged *Lmi*PGAM and subsequent cell lysis were performed as described previously. The protein was purified by metal-affinity chromatography via the His tag LEHHHHHH (Fuad et al. 2011), followed by ion-exchange chromatography (Blackburn et al. 2014). Briefly, the *Lmi*PGAM was initially passed through a 5 mL IMAC Hitrap HP column (GE Healthcare), pre-charged nickel, and after washing was eluted by 500 mM imidazole. The pooled *Lmi*PGAM fractions were applied to a 1 mL MonoQ 5/50 GL anion-exchange column (GE Healthcare) after a washing step with buffer A (20 mM HEPES (Sigma Life Sciences) pH7.6). The protein was eluted with buffer B (20 mM HEPES, 1 M NaCl (Fisher Scientific) pH7.6) in a linear gradient, for 80 column volumes and yielded three main peaks as described by Blackburn et al. (2014). These were pooled separately and buffer-exchanged into 20 mM TEA-HCl (Sigma Life Sciences) pH7.6 and 50 mM NaCl (Fisher Scientific) using a 53 mL HiPrep 26/10 desalting column (GE Healthcare). The major peak was used for activity measurements and corresponded to *Lmi*PGAM in its open conformation (Blackburn et al. 2014). The protein concentration was measured as described previously (Fuad et al. 2011). The enzyme was stored in the cold room (4°C) before being used for the subsequent inhibition assays.

### DOCKING PARAMETERS AND CONTROL EXPERIMENTS FOR VIRTUAL SCREENING

Water molecules and other hetero atoms were removed from the structures and the program PDB2PQR 1.8 (Dolinsky et al. 2007) was used to assign position-optimised hydrogen atoms, utilising the additional PropKa (Li et al. 2005) algorithm with a pH of 7.4 to predict protonation states. The MGLTools 1.5.4 utility `prepare_receptor4.py` was used to assign Gasteiger charges to atoms. Hydrogen atoms were assigned to ligand structures using OpenBabel 2.3.1 (O'Boyle et al. 2011), utilising the `-p` option to predict the protonation states of functional groups at pH7.4. The MGLTools utility `prepare_ligand4.py` was used to assign Gasteiger charges and rotatable bonds and MOPAC 2012 was used to calculate the charge of the zinc ion. As Vina 1.1.2 (Trott & Olson 2010) and Autodock 4.2.3 (Cosconati

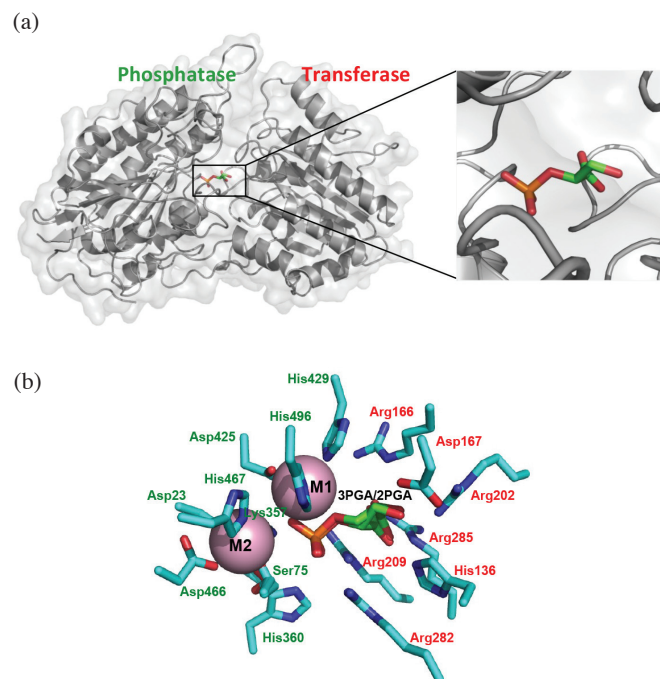


FIGURE 1. (a) The closed-form crystal structure of *LmiPGAM* (Nowicki et al. 2009; PDB ID: 3IGY) showing the phosphatase and transferase domains, and with 2PGA in the active site. (b) The 16 residues which are conserved in the active site. The green text indicates residues in the phosphatase domain, whereas the red text indicates residues in the transferase domain. The spheres labelled M1 and M2 correspond to the divalent metal-binding sites which are occupied by  $\text{Co}^{2+}$  in the crystal structure

et al. 2010; Huey et al. 2007) both use the same .pdbqt format for their input, the same prepared files could be used for each. A grid box that encompassed the maximum dimensions of the ligand plus 12 Å in each direction was used. The starting translation and orientation of the ligand and the torsion angles of all rotatable bonds were set to random. The Autogrid grid point spacing was set at 0.2 Å. The Autodock parameter file specified 10 Lamarckian genetic algorithm runs, 2,000,000 energy evaluations and a population size of 300. Each docking program was used to automatically dock the cognate ligand into the 2PGA binding pocket of the crystal structure of the closed-form of *LmiPGAM* (PDB: 3IGY).

#### CURATION OF THE VIRTUAL CHEMICAL LIBRARY

The screening compound stock lists in SDF format of ChemBridge, Asinex, Maybridge, Enamine, LifeChemicals, Specs, InterBioScreen, ChemDiv and KeyOrganics were merged. Salts were stripped out using Sieve 3.1.0 (de Winter 2014) and duplicates removed using canonical SMILES string comparison via Open Babel 2.3.1. The supplied 2D coordinates were converted into 3D using Concord 4.08. Because the aim was to identify initial lead-like hits suitable for optimisation into more drug-like molecules, the virtual library was filtered according to Oprea 'lead-like' rules (H-bond acceptors  $\leq 9$ ; H-bond donors  $\leq 5$ ; MW  $\leq 460$ ; cLogP between -4.6 and 4.2; cLogS  $\geq -5$ ; Number of rings  $\leq 4$ ; Number of rotatable bonds  $\leq 9$ ) (Hann & Oprea 2004). This left 1,137,587 molecules. A multi-conformer version of this virtual library was

produced using Multiconf-DOCK (Sauton et al. 2008); an average of 4.25 conformers per compound were generated depending on flexibility; this resulted in a virtual library containing a total of 4,840,093 conformers.

#### VIRTUAL SCREENING

An initial ligand-based pharmacophore screen was carried out. 2PGA from the crystal structure was used as a search term. The programs UFSRAT (Shave et al. 2015), ROCS 3.0.0 (Hawkins et al. 2007) and a Wiener index similarity comparison algorithm available as part of EDULISS 2.0 (Hsin et al. 2011) were used to search the virtual library for molecules with different types of similarity to the known ligands. The UFSRAT search of the multi-conformer library returned 7,268 molecules, ROCS returned 10,002 and EDULISS 10,000. UFSRAT was accessed through the Centre for Translational and Chemical Biology, The University of Edinburgh website (<http://opus.bch.ed.ac.uk/ufsrat/>).

In addition, the rigid-body docking program LIDAEUS (Taylor et al. 2008) (<http://opus.bch.ed.ac.uk/lidaeus/>) was used to dock the conformer virtual library into the 2PGA binding site of the *LmiPGAM* crystal structure. The results were ranked according to LIDAEUS score, the top 28,757 compounds from this list merged with the results from the ligand-based methods described above and the duplicates removed. This resulted in 21,603 unique molecules which were docked into *LmiPGAM* using Vina. Docked poses were scored using both Vina's internal scoring algorithm and X-Score 1.2 (Wang et al. 2002); these scores were used via a 'rank-by-rank' consensus scheme (Wang & Wang



2001) to create a ranked list. The top 200 compounds were then used as search terms for another iteration of the ligand-based virtual screening methods as described above. This resulted in 7,386 unique molecules, which were also docked using Vina. The top 1,920 of these were docked using Autodock, of which 1,196 predicted binding modes matched those of Vina (coordinate root-mean-square deviation < 2.0 Å). These 'consensus docked' (Houston & Walkinshaw 2013) molecules were also scored using DrugScore 1.2 (Gohlke et al. 2000). A final ranked list was prepared via rank-by-rank scheme, taking the Vina, Autodock, X-Score and DrugScore scores into account.

#### QUANTITATIVE HIGH-THROUGHPUT SCREENING

Quantitative high-throughput screening (qHTS) was performed by using the Kinase-Glo Plus® reagent (Promega), which quantifies the amount of ATP in a sample following a kinase reaction. A 1536-well plate-based assay was used in the glycolytic direction with purified *LmiPGAM*, with the addition of enolase (ENO) and pyruvate kinase (PYK) as coupling enzymes and was done in the presence of 0.13 mM CoCl<sub>2</sub>. The 1280 compound Library of Pharmacologically Active Compounds (LOPAC®) (Sigma-Aldrich) was used for a preliminary screen. A 7-point dilution series for each compound was used and the enzymes and compounds were incubated for 60 min prior to the addition of the Kinase-Glo reagent (<http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=504639>).

#### IN VITRO INHIBITION ANALYSIS WITH SELECTED COMPOUNDS

A preliminary screen was performed according to the plate-based assay for the activity of iPGAM coupled with the oxidation of NADH to NAD<sup>+</sup> by lactate dehydrogenase (LDH) in the presence of ENO and PYK as reported previously (Fuad et al. 2011). A final concentration of 0.01 mg/mL *LmiPGAM* was incubated with a final concentration of 1 mM CoCl<sub>2</sub> at 25°C for 1 h, before 1 mM of compounds was added, followed by a further 1 h incubation. The reaction was started by the addition of 0.01 mg/mL iPGAM that has been incubated with 1 mM CoCl<sub>2</sub> and selected compounds into the reaction mixture containing 100 mM TEA-HCl buffer pH7.6, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM NADH, 1 mM ADP, 1 unit of ENO, 2 units of PYK and 3 units of LDH (1 unit of enzyme converts 1.0 μmole of substrate per min at pH7.4 at 25°C). The assays were done in triplicate. All stock solutions of the compounds were prepared by dissolving them with 100% DMSO, except for compounds 5 and 10, which were dissolved with 50% DMSO, before being further diluted with 200 mM TEA-HCl pH7.6 to prepare a 10 mM stock solution (Fuad 2012). Compound 1 was from Enamine (Kiev, Ukraine) and compounds 2 and 3 from Sigma-Aldrich. Compounds 4-7 were from Enamine and compounds 8-11 from InterBioScreen (Moscow, Russia). Cobalt was the divalent metal used in the assay because it hyperactivates *LmiPGAM* *in vitro* (Fuad et al. 2011). The presence of residual Ni<sup>2+</sup> in purified *LmPGAM* from the

metal-affinity column was regarded as unlikely because the enzyme had experienced ion-exchange and gel filtration column purification steps (in the absence of added divalent metals) after the metal-affinity column step.

## RESULTS AND DISCUSSION

### UFSRAT IDENTIFIES SMALL MOLECULE ANALOGUES OF 2PGA/3PGA

The closed-form crystal structure of *LmiPGAM* comprises two distinct domains that play individual roles in the catalytic function of the enzyme. Two Co<sup>2+</sup> ions were coordinated within the active site, together with both substrate and product, which were present at equal occupancies (Figure 1(a)) (Nowicki et al. 2009). It can be seen that residues from both the phosphatase and transferase domains contribute to ligand binding (Figure 1(b)). The small and poorly accessible active site makes the prediction of interactions with drug-like compounds difficult. UFSRAT has the ability to identify molecules that are similar to the substrate 3PGA or product 2PGA (Figure 2), thus increasing the likelihood of discovering small molecule inhibitors that may be inserted into the active site of *LmiPGAM*.

The overall shape similarities were not the only consideration in the UFSRAT algorithm, as it also examines the types and molecular topology of the existing atoms. The geometrical distribution of atoms was divided into four categories based on all atom types, hydrophobicity, hydrogen acceptor, or hydrogen donor atoms (Fuad 2012; Shave et al. 2015). Taking these descriptors into consideration and using 3PGA/2PGA as the query molecules, a search was carried out, examining the contents of a large multi-conformer library comprising 4,853,000 molecules from various suppliers (Chembridge, Asinex, Maybridge, Enamine, LifeChemicals (InfLab) Specs, InterBioScreen and ChemDiv) to identify candidates similar to the query molecules. In this process, similarity was evaluated through the scoring function, where 0 indicates the least similar compounds, whereas a score near to 1 suggests a close resemblance. The scores associated with the five compounds most closely resembling 3PGA ranged from 0.739 to 0.770, while the scores of compounds similar to 2PGA ranged from 0.708 to 0.728 (data not shown).

### MOLECULAR DOCKING FOR STRUCTURE-BASED VIRTUAL SCREENING

Molecular docking was employed using both AutoDock 4 and AutoDock Vina to generate a grid map based on the active site region. The map was subsequently used to screen approximately 200 compounds obtained from UFSRAT. The predicted binding affinity was calculated for all 200 compounds using an algorithm that was applied in both programmes. The results, in general, met the criteria that are commonly accepted by Lipinski's rule of 5 for molecule drug-likeness (Lipinski et al. 1997; Taylor et al.

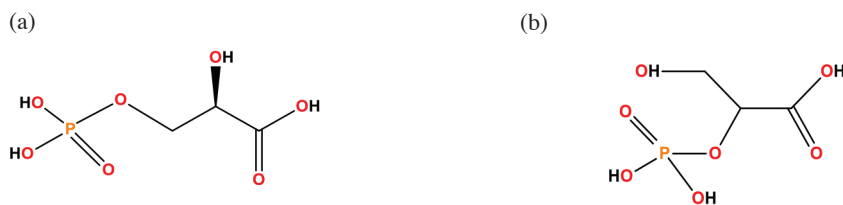


FIGURE 2. The query molecules which were used for UFSRAT: (a) 3PGA and (b) 2PGA

2008). Both the AutoDock4 and AutoDock Vina results were individually assessed in terms of the scores, which were based on the binding affinities. Strong interactions were indicated by negative and low free energy of binding ( $\Delta G_{\text{bind}}$ , kcal/mol) values, which also signified that the potential inhibitor was in its most favourable conformation (Messaoudi et al. 2013).

A proprietary combinatorial virtual screening scheme, which is still under development at Edinburgh University, was also used to obtain comparable and accurate results. This workflow combines both similarity searches and docking algorithms to provide a consensus prediction of possible molecular interactions. This process involved a cycle of parallel fast flexible docking and similarity search algorithms comprising AutoDock, AutoDock Vina and LIGand Discovery At Edinburgh (LIDAEUS), which were employed against the Edinburgh University LIGand Selection System (EDULISS) database. It is relevant to mention that  $\text{Zn}^{2+}$  atoms were included in this analysis, as it was reported that  $\text{Zn}^{2+}$  might be the biologically relevant metal for trypanosomatid iPGAM (Fuad et al. 2011). Compounds from the three programs were evaluated based on the binding affinity, solubility, range of chemical classes covered and specific chemical groups contained, which may resemble the properties of the substrate and product. Eight of these compounds (Figure 3) were subsequently selected for the inhibitory analysis.

#### ANALYSIS OF POTENTIAL INHIBITORS

In parallel with the virtual screening work (which yielded the eight compounds in Figure 3), three additional compounds were selected from preliminary qHTS of *Lmi*PGAM using the LOPAC<sup>®</sup> library (<http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=504639>) and are shown in Figure 4. The eleven compounds thus identified by both screening methods were tested for inhibitory activity. Their structures are shown in Figures 3 and 4.

The enzyme assay described in materials and methods was used and the potential inhibitory effects on the coupling enzymes (ENO, PYK and LDH) were assessed in the absence of *Lmi*PGAM when the assay reaction included 2PGA instead of 3PGA. The concentration of each compound was fixed at 1 mM and the inhibitory effect was expressed as the percentage of remaining activity of *Lmi*PGAM after the addition of the compounds (Figure 5). Of the 11 compounds, two showed substantial specific inhibition of *Lmi*PGAM (compound 2 from the LOPAC<sup>®</sup> screen, 86% inhibition; compound 7 from the virtual screen, 51% inhibition). It can be noted that the inhibitory effect of compound 3 seemed to act at the level of one or more of the coupling enzymes and not specifically on *Lmi*PGAM. The assay with compound 9 showed no absorbance at 340 nm, indicating that the NADH in the assay had been completely converted to NAD<sup>+</sup>. This effect was observed in both enzyme samples and was therefore not specific for *Lmi*PGAM. Compounds 4 and 5 from

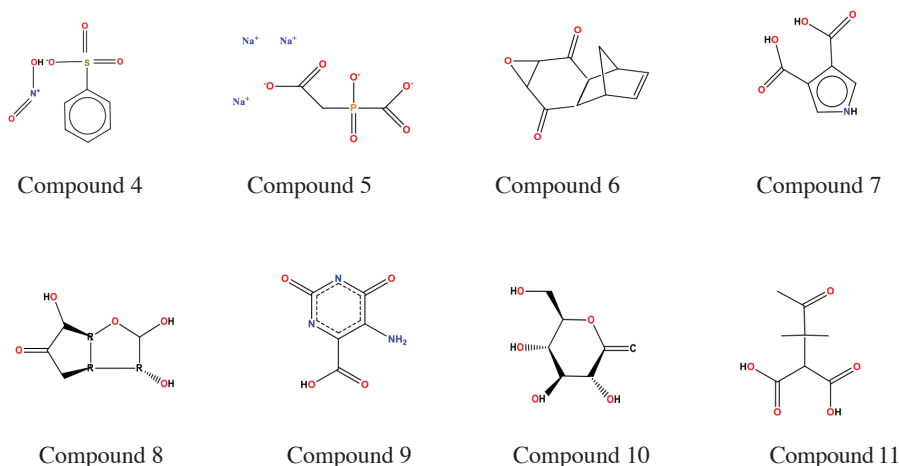


FIGURE 3. A compilation of compounds identified from the structure-based virtual screens

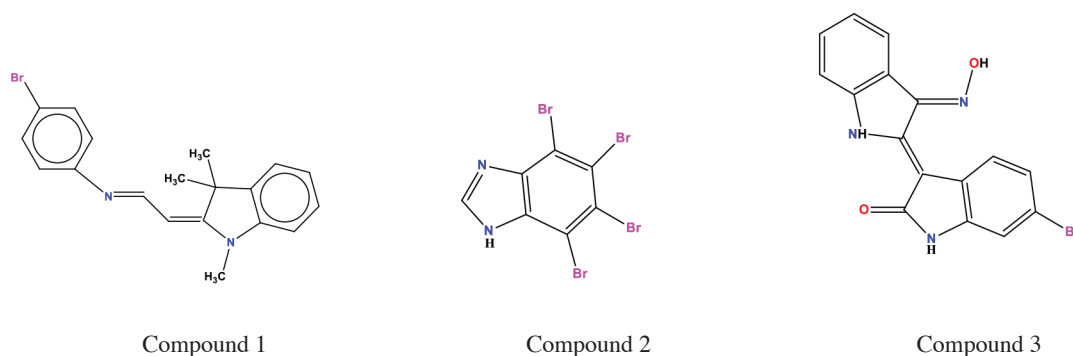


FIGURE 4. Compounds identified by high-throughput screening

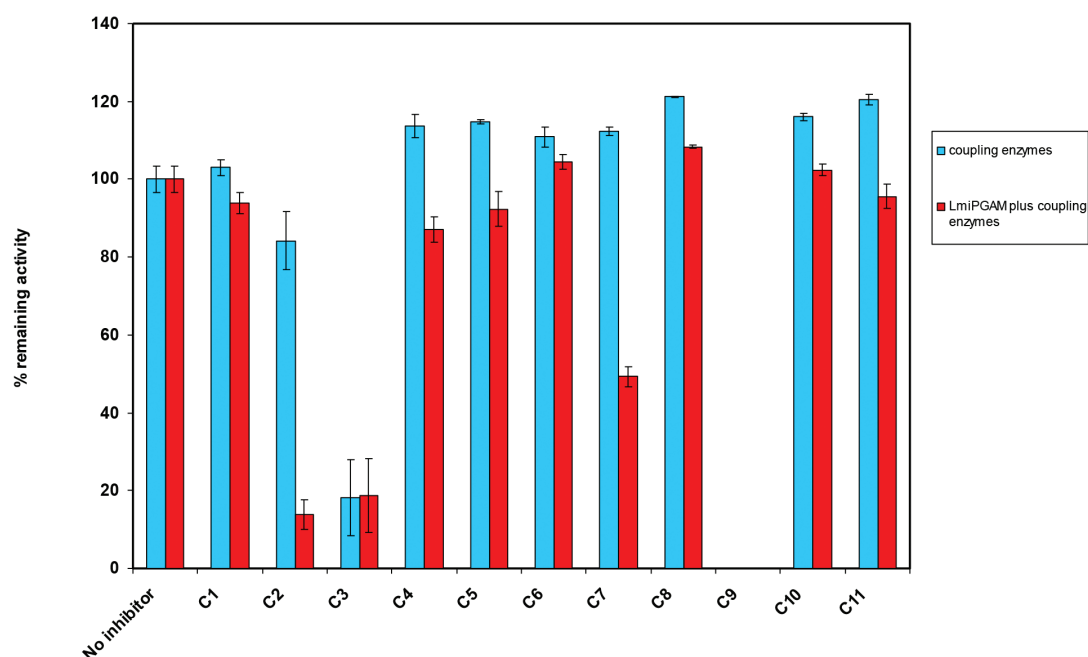


FIGURE 5. Inhibition analysis for *LmiPGAM* plus coupling enzymes (red bars) and for the coupling enzymes alone (blue bars). 3PGA or 2PGA were used as the starting reagents for the two assays, respectively. The assays were carried out with 1 mM of each compound as described in materials and methods. The assay with compound 9 showed no absorbance at 340 nm

the virtual screen showed modest specific inhibition of *LmiPGAM*.

No potent inhibitors of trypanosomatid iPGAMs have previously been reported other than non-specific metal chelators. Thus, an important implication of our findings was the potential for some of the compounds we identified to be developed as specific inhibitors for *LmiPGAM*. Moreover, the structural and mechanistic similarities between *LmiPGAM* and *TbiPGAM* mean that inhibitors of the former enzyme may well be relevant to inhibition of the latter. This is of special significance in the context of the recent publication of the results of two high-throughput screens of iPGAM from the nematodes *Brugia malayi* and *Caenorhabditis elegans* (Crowther et al. 2014). They concluded that iPGAM has limited druggability. However, it is relevant to note that nematode and trypanosomatid iPGAMs belong to different families, with different

mechanisms of opening and closing their two-domain structures, as well as different closed structures (Blackburn et al. 2014). Furthermore, the recent description of a spring-loaded mechanism inherent to the iPGAM structure which favours the open conformation in the absence of ligands is also of crucial importance (Roychowdhury et al. 2015). In the context of drug discovery, all these observations add up to encourage the use of virtual screening on the one hand and on the other of using enzyme assay conditions that may facilitate ligand binding, such as extended pre-incubation periods.

## CONCLUSION

Making use of both virtual and high-throughput screens, we have identified several inhibitors for *LmiPGAM*, which could serve as starting points for drug discovery

for trypanosomatid iPGAMS. The results showed that two compounds gave substantial inhibition of 86% (compound 2) and 51% (compound 7) at 1 mM. Interestingly, compound 7 was discovered from the virtual screen, whereas compound 2 was one of the compounds obtained from HT screening.

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