Leishmaniasis are neglected tropical diseases that threaten about 350 million people in 98 countries around the world. In order to find new antileishmanial drugs, an original approach consists in reducing the pathogenic effect of the parasite by impairing the glycoconjugate biosynthesis, necessary for parasite recognition and internalization by the macrophage. Some proteins appear to be critical in this way, and one of them, the GDP-Mannose Pyrophosphorylase (GDP-MP), is an attractive target for the design of specific inhibitors as it is essential for *Leishmania* survival and it presents significant differences with the host counterpart. Two GDP-MP inhibitors, compounds A and B, have been identified in two distinct studies by high throughput screening and by a rational approach based on molecular modeling, respectively. Compound B was found to be the most promising as it exhibited specific competitive inhibition of leishmanial GDP-MP and antileishmanial activities at the micromolar range with interesting selectivity indexes, as opposed to compound A. Therefore, compound B can be used as a pharmacological tool for the development of new specific antileishmanial drugs.

**Keywords:** GDP-mannose pyrophosphorylase, *Leishmania*, therapeutic target, inhibitors, drug development

**INTRODUCTION**

Leishmaniasis are vector-borne neglected tropical diseases caused by a protozoan parasite from the genus *Leishmania* and transmitted by hematophagous female phlebotomine sandflies. During its life cycle, the parasite alternates from a promastigote motile form within the phlebotome to an intracellular amastigote form in mammalian host macrophages. Leishmaniasis can be classified in three main groups according to their clinical manifestations: cutaneous, which is the most common form, mucocutaneous leading to nasal and oropharyngeal lesions and marked disfigurements, and visceral, the most severe form, always fatal in the absence of adequate treatment. These clinical manifestations can be provoked by several *Leishmania* species: for instance, *L. major* or *L. mexicana* will give rise to cutaneous leishmaniasis, and *L. donovani* or *L. infantum* visceral leishmaniasis. Only few drugs are currently available for the treatment of leishmaniasis. Antimonials, which have been historically used since 1920s, generate a strong toxicity at cardiac, renal, and hepatic levels and select drug resistance. The other classical drugs, namely oral miltefosine, injectable liposomal amphotericin B, and paromomycin, display some deleterious effects and now represent a potential threat of drug resistance as well (Croft et al., 2006; Sundar and Singh, 2016; Ponte-Sucre et al., 2017). The development of new antileishmanial treatments is thus crucial in this context.
In order to overcome the limitations of the existing treatments, rational approaches have been used to develop new specific therapies for leishmaniases (Zulfiqar et al., 2017). Among the different strategies elaborated, the identification of new targets that are essential for parasite viability or virulence is an attractive approach for the development of specific antileishmanial compounds (Jiang et al., 1999; Burchmore et al., 2003; Jain and Jain, 2018). Indeed, these essential targets can be exploited by chemical screening in order to characterize inhibitor scaffolds whose specificities are optimized by pharmacomodulations, based on target three-dimensional structures. In this way, targets from Leishmania energy metabolism (i.e., glycolysis, folate or redox metabolism) were first intensively studied (Aronov et al., 1999; Chowdhury et al., 1999; Verlinde et al., 2001; Olin-Sandoval et al., 2010; Colotti et al., 2013; Leroux and Krauth-Siegel, 2016). Other biochemical pathways were also investigated, but the characterized inhibitors met some limitations such as parasite specificity, inhibitors synthesis cost and lack of in vivo activity (Croft and Coombs, 2003).

TARGETING MEMBRANE GLYCOCONJUGATE METABOLISM

There are two main ways to impair parasite development within the host, considering proteins expressed in the amastigote form as therapeutic targets. The first one relies on targeting some biochemical pathways leading to an unbalanced metabolism, toxic for the parasite. Many proteins have been considered for this purpose (Aronov et al., 1999; Chowdhury et al., 1999; Verlinde et al., 2001; Olin-Sandoval et al., 2010). The second one considers that a relevant Achilles heel consists in avoiding macrophage-parasite interactions (Descoteaux et al., 1995; Descoteaux and Turco, 1999; Podinovskaia and Descoteaux, 2015; Lamotte et al., 2017). As host-Leishmania interactions mainly rely on glycoconjugate recognition, an inhibition of glycoconjugate biosynthesis could affect this molecular recognition, and therefore reduce parasite burden. Furthermore, as the glycosylation is a crucial pathway for macrophage infection (Descoteaux et al., 1995; Descoteaux and Turco, 1999; Pomel and Loiseau, 2013; Podinovskaia and Descoteaux, 2015), we hypothesize that an alteration of glycoconjugate structures would not easily select drug resistance.

Mannose-containing glycoconjugates represent a large proportion of the carbohydrates addressed at the surface of a eukaryotic cell and are involved in many biological processes such as intercellular recognition, adhesion or signaling (Varki, 2007; Colley et al., 2017). In Leishmania, a wide range of unusual mannose-containing glycoconjugates [e.g., GlycosylPhosphatidylinositol (GPI) anchors, LipoPhosphoGlycans (LPG), ProtoePhosphoGlycans (PPG) or GlycosylInositolPhosphoLipids (GIPLs)] are synthesized and are essential for parasite virulence (Descoteaux and Turco, 1999; Pomel and Loiseau, 2013). The biosynthesis of these glycoconjugates requires initially the conversion of mannose into GDP-mannose. The mannose moiety of this nucleotide sugar is then transferred into nascent glycoconjugates to allow mannosylation reaction. In eukaryotic cells, mannose can either be imported via membrane transporters or be generated from the reaction catalyzed by the PhosphoMannose Isomerase (PMI) on fructose-6-phosphate originating from glycolysis to produce mannose-6-phosphate (Figure 1A). In the mannosylation pathway, the PhosphoMannomutase (PMM) converts mannose-6-phosphate in mannose-1-phosphate (Figure 1). The activated form of mannose, GDP-mannose, is then produced by the action of the GDP-Mannose Pyrophosphorylase (GDP-MP) according to the following reversible enzymatic reaction (Ning and Elbein, 2000):

\[
\text{Mannose} - 1 - P + GTP \rightleftharpoons GDP - \text{Mannose} + PPi
\]

The GDP-MP is a ubiquitous enzyme found in bacteria, fungi, plants, and animals and belonging to the family of nucleotidyltransferases. In mammalian organisms, GDP-MP was mainly studied in swine (Suzumilo et al., 1993; Ning and Elbein, 2000). The swine native enzyme is a complex of about 450 kDa with two distinct subunits: α (43 kDa) and β (37 kDa). In pig, as well as in human, the β subunit displays the enzymatic activity, while the α subunit would have a regulatory function (Suzumilo et al., 1993; Ning and Elbein, 2000; Carss et al., 2013; Koehler et al., 2013). In human, α and β subunits share 32% identity. Mutations in the genes coding for α or β subunits in human lead to glycosylation disorders characterized notably by neurological deficits and muscular dystrophies (Carss et al., 2013; Koehler et al., 2013). Two β isoforms, named β1 and β2, have been characterized in the human genome, displaying 90 and 97% identity with the porcine β subunit, respectively. The human β2 isoform is strongly expressed in a wide range of tissues, in opposition to β1 which is only weakly expressed, especially in liver, heart, and kidney (Carss et al., 2013). Additionally, the β2 isoform shows a better homology with Leishmania mexicana GDP-MP, compared to β1 (49% for β2 vs. 46% for β1). In bacteria, GDP-MP are mostly dimeric, either mono- or bifunctional, the latter displaying both GDP-MP and PMI activities in separate domains of an individual enzyme (Shinabarger et al., 1991; May et al., 1994; Ning and Elbein, 1999; Wu et al., 2002; Asencion Diez et al., 2010; Pelissier et al., 2010; Akutsu et al., 2015). Unlike in other organisms, leishmanial GDP-MP has been shown to assemble as a hexamer of 240 kDa in several Leishmania species (Davis et al., 2004; Mao et al., 2017). As this hexamer can dissociate at low ionic strength conditions and at low protein concentration, a mixture of the three forms may be present in the reaction medium in vitro.

Both human and leishmanial GDP-MP have been reported to display a high substrate specificity (Mao et al., 2017), in agreement with previous studies performed in bacterial, trypanosomal, and swine GDP-MP (Ning and Elbein, 2000; Denton et al., 2010; Pelissier et al., 2010). The investigation of the mechanism of reaction has shown a sequential ordered mechanism in most bacterial GDP-MP like in some other
FIGURE 1 | Mannose activation pathways and GDP-MP inhibitors. (A) Mannose activation pathways and glycoconjugate biosynthesis in Leishmania. The GDP-MP substrates and products are indicated in blue. The GDP-MP is circled in red. (B) Chemical structures of compounds A,B. (C) Docking analyses of compound A (top) and B (bottom) in LmGDP-MP (left) and hGDP-MP (right) catalytic sites. The protein surface is colored as a function of the charge density: red, white, and blue colors indicating negative, neutral, and positive area, respectively. Magnesium ion is represented by a green sphere. The amino acids that make contact with compound A,B in the catalytic sites are indicated in their one-letter code and number in the sequence (Adapted from Daligaux et al., 2016a; Mao et al., 2017).

nucleotidyl-transferases, with GTP fixation prior to mannose-1-phosphate (Barton et al., 2001; Zuccotti et al., 2001; Asencion Diez et al., 2010; Pelissier et al., 2010; Boehlein et al., 2013). However, leishmanial and human GDP-MP have been characterized by a sequential random mechanism (Mao et al., 2017), in which the substrate binding order is not defined, in agreement with a mammalian nucleotidyl-transferase (Persat et al., 1983), suggesting that the GDP-MP mechanism of reaction differs from bacteria to Leishmania and human.

A knockout of the gene encoding for GDP-MP in L. mexicana lead to an absence of development in the macrophage in vitro and to an absence of parasite persistence in vivo (Garami and Ilg, 2001; Stewart et al., 2005). These results show that GDP-MP is critical for amastigote survival and is therefore an interesting drug therapeutic target to be exploited for antileishmanial drug development. Likewise, GDP-MP has been described to be essential for cell integrity and survival in other microorganisms such as Trypanosoma brucei, Aspergillus fumigatus, or Candida albicans showing the biological validation as a potential therapeutic target of this enzyme in several kinetoplastids and fungi (Warit et al., 2000; Jiang et al., 2008; Denton et al., 2010). Additionally, a High-Throughput Screening (HTS) assay, allowed the selection of leishmanial GDP-MP inhibitors (Lackovic et al., 2010). From this study, the most potent inhibitor identified was a piperazinyl quinoline derivative (compound A; Figure 1B) demonstrating an in vitro activity on L. major GDP-MP and on intracellular parasite proliferation with IC_{50} values at 0.58 and 21.9 µM, respectively.

COMPUTATIONAL AND TARGET-BASED DRUG DESIGN

A molecular model of the GDP-MP quaternary structure has been generated in L. mexicana, confirming the hexameric structure of the enzyme (Perugini et al., 2005). Based on this model, GDP-MP hexamers would be assembled by a contact between trimer structures in a head-to-head manner involving only the N-terminal end of the protein. These results are however in opposition to crystallography studies of other GDP-MP or nucleotidyl-transferases, showing a tail-to-tail arrangement of the C-terminal β-helices in their quaternary structures (Cupp-Vickery et al., 2005; Jin et al., 2005; Pelissier et al., 2010; Führing et al., 2015).
As no GDP-MP crystal could be obtained in *Leishmania*, molecular models of *L. infantum* and *L. donovani* GDP-MP were generated using distinct sequence alignment strategies and were compared with the human counterpart (Pomel et al., 2012; Daligaux et al., 2016a). Both analyses showed a structural conservation of a consensus sequence GXGXRX₆K in leishmanial and human GDP-MP corresponding to a pyrophosphorylase signature motif, as well as the F(V)EKP sequence previously described to be part of the GDP-MP active site (Sousa et al., 2008). Interestingly, several specific residues have been identified in the catalytic site of both *L. infantum* and *L. donovani* GDP-MP compared to the human counterpart (Pomel et al., 2012; Daligaux et al., 2016a). Moreover, GDP-MP sequences share more than 85% of identity in the *Leishmania* genus. Therefore, the differences identified between the leishmanial and human catalytic sites could potentially be exploited to design specific antileishmanial agents.

The GDP-mannose, as a substrate or a product of the GDP-MP, has been selected as the basis for inhibitor design because of its steric volume presenting the maximum of interactions within the enzyme catalytic pocket (Mao et al., 2017). In this work, the chemical approach to design leishmanial GDP-MP inhibitors relied on the pharmacomodulation of the GDP-mannose from the analysis of enzyme molecular models, by substituting for example the mannos moieties by a phenyl group, the pyrophosphate by a triazole or a phosphate, the ribose by an ether oxide group or a deoxyribose and the guanine by different heterocycles such as purine analogs or quinolines, especially two-substituted quinolines which have been previously described to display promising *in vitro* and *in vivo* antileishmanial activities (Fournet et al., 1993, 1994, 1996; Nakayama et al., 2005, 2007; Campos-Vieira et al., 2008; Loiseau et al., 2011). Therefore, the presence of two-substituted quinolines in these compounds designed could potentiate their antileishmanial activities through GDP-MP inhibition.

**CELL-FREE IN VITRO AND IN SILICO EVALUATION OF COMPOUNDS ON PURIFIED GDP-MPs**

From the analysis of GDP-MP structural models, a library of 100 compounds was designed and synthesized (Daligaux et al., 2016b; Mao et al., 2017). These compounds were evaluated on recombinant GDP-MP purified from *L. donovani* (LdGDP-MP), *L. mexicana* (LmGDP-MP), and human (hGDP-MP). In this work, the hGDP-MP corresponded to the β2 subunit displaying the enzyme activity and showing the highest homology with leishmanial GDP-MP (see above). This evaluation allowed to identify compound B, a quinoline derivative substituted in position 2 with a methoxy-ethyl-triazol-buty-diisopropylphosphonate group (Figure 1B), as a specific competitive inhibitor of LdGDP-MP with a $K_i$ at 7 µM. In comparison, compound A, previously identified from a HTS (Lackovic et al., 2010), displayed a competitive inhibition of both LdGDP-MP and hGDP-MP with $K_i$ values at 62 and 20 µM, respectively, reflecting a lower affinity for the leishmanial enzyme compared to the human counterpart.

A docking study of the identified competitive inhibitors on GDP-MP structural models showed that compound A binds to both LdGDP-MP and hGDP-MP with similar potency and binding modes: the quinoline, piperazine, and tert-butyl groups occupying the same position as the GDP-mannose nucleotide, ribose and mannose moieties, respectively, in both catalytic sites (Daligaux et al., 2016a; Figure 1C). In contrast, compound B was found to bind more strongly to LdGDP-MP compared to hGDP-MP, with the diisopropylphosphonate group located more deeply in the leishmanial enzyme catalytic pocket compared to the human one (Mao et al., 2017; Figure 1C). These *in silico* data are in agreement with the non-selective inhibition of both leishmanial and human GDP-MP by compound A and the specific competitive inhibition observed with compound B on LdGDP-MP.

**CELLULAR IN VITRO ANTILEISHMANIAL ACTIVITY AND CYTOTOXICITY OF COMPOUNDS A AND B**

Both compounds have been evaluated on *L. donovani* and *L. mexicana* axenic and intramacrophage amastigotes in two host cell models: the RAW264.7 macrophage cell line and primary Bone Marrow Derived Macrophages (BMDM; Mao et al., 2017). Compound A showed a moderate antileishmanial activity on both *L. mexicana* and *L. donovani* with IC₅₀ values between 30 and 50 µM and between 12 and 28 µM on axenic and intramacrophage amastigotes, respectively (Mao et al., 2017; Table 1). These data are in agreement with the non-selective inhibition of both leishmanial and human GDP-MP by compound A and the specific competitive inhibition observed with compound B on LdGDP-MP.
| Compounds                  | L. donovani | L. mexicana | L. donovani | L. mexicana |
|---------------------------|-------------|-------------|-------------|-------------|
|                           | axenic amastigotes | infected RAW.264.7 macrophages | infected BMDM | infected RAW.264.7 macrophages | infected BMDM | infected RAW.264.7 macrophages | infected BMDM | infected RAW.264.7 macrophages |
| A                         | 30.68 ± 6.62 | 19.52 ± 4.53 | 12.18 ± 4.74 | 12.05 ± 2.23 |
| B                         | 1.06 ± 0.10  | 0.83 ± 0.14  | 1.06 ± 0.31  | 0.79 ± 0.26  |

The table was adapted from Mao et al. (2017). The results expressed correspond to the mean of three independent experiments (± SD).

In conclusion, compound B can be considered as an original and interesting hit to be optimized proving that GDP-MP inhibition is a promising strategy to impair host-parasite interactions. However, the capacity of this specific metabolism alteration to prevent drug resistance emergence is still to be proved.

AUTHOR CONTRIBUTIONS

SP wrote the manuscript. WM, TH-D, CC, and PL contributed to manuscript revision and read and approved the submitted version.

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