Targeting UDP-Galactopyranose Mutases from Eukaryotic Human Pathogens

Karina Kizjakina¹, John J. Tanner²,³,* and Pablo Sobrado¹,*

¹Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA; Departments of ²Chemistry and ³Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

Abstract: UDP-Galactopyranose mutase (UGM) is a unique flavin-dependent enzyme that catalyzes the conversion of UDP-galactopyranose (UDP-Galp) to UDP-galactofuranose (UDP-Galf). The product of this reaction is the precursor to Galf, a major component of the cell wall and of cell surface glycoproteins and glycolipids in many eukaryotic and prokaryotic human pathogens. The function of UGM is important in the virulence of fungi, parasites, and bacteria. Its role in virulence and its absence in humans suggest that UGM is an ideal drug target. Significant structural and mechanistic information has been accumulated on the prokaryotic UGMs; however, in the past few years the research interest has shifted to UGMs from eukaryotic human pathogens such as fungi and protozoan parasites. It has become clear that UGMs from prokaryotic and eukaryotic organisms have different structural and mechanistic features. The amino acid sequence identity between these two classes of enzymes is low, resulting in differences in oligomeric states, substrate binding, active site flexibility, and interaction with redox partners. However, the unique role of the flavin cofactor in catalysis is conserved among this enzyme family. In this review, recent findings on eukaryotic UGMs are discussed and presented in comparison with prokaryotic UGMs.

Keywords: UDP-galactopyranose mutase, enzyme drug target, galactofuranose, non-redox reaction, flavoenzyme, galactopyranose, inhibitors, eukaryotic pathogens.

1. INTRODUCTION

Vector-borne diseases like Chagas disease and leishmaniasis are caused by parasitic human pathogens and are a major health burden in many developing countries. Current therapies are not very effective and suffer from toxic side effects [1-2]. In addition, the emergence of drug-resistant strains has been reported [3-6]. These vector-borne diseases have been recognized by the World Health Organization (WHO) as Neglected Tropical Diseases (NTD) – chronic infectious diseases endemic mainly in underdeveloped countries, and even though millions of people are affected and thousands die every year, there are no effective cures [7]. In recent years, significant research efforts have been focused on NTD due to policies and research programs implemented by the WHO and other governmental and private organizations [8]. Fungi from Aspergillus species cause a series of broncho-respiratory infections collectively known as aspergillosis [9-10]. Infections by Aspergillus fumigatus are the most common in immuno-compromised individuals. Once infection has been established, the mortality rate can be close to 50% [11]. Therefore, new effective anti-fungal drugs are urgently needed.

A possible mode of intervention against these parasitic and fungal pathogens is to inhibit the activity of enzymes that aid in cell wall biosynthesis and/or host-pathogen interactions [12-13]. It has recently been shown that galactofuranose (Galf), a sugar not found in humans, plays an important role in cell wall biosynthesis in A. fumigatus and many bacteria and is a major component of the cell surface matrix of Trypanosoma cruzi and Leishmania major, the causative agents of Chagas disease and leishmaniasis, respectively. In these parasites, Galf plays a major role in virulence [14]. The flavoenzyme UDP-galactopyranose mutase (UGM) is a unique enzyme not present in humans and is essential in the biosynthesis of Galf. Here, we provide an overview of the biosynthesis of Galf and its role in pathogenesis in eukaryotic pathogens with a focus on recent studies on eukaryotic UGMs from T. cruzi, L. major, and A. fumigatus.

1.1. Neglected Tropical Diseases Caused by T. cruzi and L. major

Chagas disease (or American trypanosomiasis) is endemic throughout Central and South America. It is caused by the protozoan parasite T. cruzi and is usually transmitted through a sylvatic cycle from an infected triatomine (“kissing bug”) vector that lays parasite-laden feces on wounds and mucous membranes, as well as in conjunctivas [15]. In addition, there have been reports of infection via blood transfusion and orally through ingesting infected mother’s milk, raw and undercooked meat, or other food infected by triatominids and/or their feces [16]. Very often in its early stages, Chagas disease is asymptomatic. If left untreated, parasite invasion becomes a serious health risk; symptoms can develop 10-20 years later when the disease becomes chronic and has high mortality rates, typically due to the parasitosis of the heart, causing myocarditis [17]. It is estimated that approximately 16-18 million people have Chagas disease and approximately 50,000 of them die annually; however, these numbers could be higher, since infections are often misdiagnosed due to the very limited, or sometimes complete lack of symptoms [15, 18].

Leishmaniasis is a vector-borne systemic disease caused by a trypanosomatid protozoa from the Leishmania spp., which invade human macrophages and replicate intracellularly after being transmitted to humans by infected sandflies (genera Phlebotomus and Lutzomyia) [19]. Depending on the particular parasitic species, leishmaniasis can develop into three forms: cutaneous, mucocutaneous, or visceral leishmaniasis [20]. L. major is the causative agent of cutaneous leishmaniasis, which manifests as a severe skin infection that often causes disfigurement and is endemic in developing countries in the tropics, sub-tropics, and the Mediterranean basin, with thousands reported new cases annually [7].

1.2. Infections Caused by A. fumigatus

Fungi of the genus Aspergillus are responsible for several human diseases ranging from allergic reactions and lung infections to sepsis and death [9]. There are hundreds of members of the Aspergillus genus, and some are pathogenic to humans, with A. fumigatus and A. niger being the most common [21-23]. Among the diseases related to Aspergillus infection, allergic bronchopulmonary aspergillosis (ABPA) and invasive pulmonary aspergillosis (IPA) represent a significant health threat to both immuno-competent and im-
muno-compromised persons [9, 24], IPA infections are commonly observed in patients receiving chemotherapy, organ transplants, and in late-stage AIDS [25-26]. An increase from 0.3% to 5.8% in IPA infections in patients admitted to intensive care units (ICUs) has been reported in recent years, and has been accompanied by a high mortality rate (50-70%) [27-28]. This demonstrates the need for new anti-fungal drugs to combat *Aspergillus* infections.

### 2. ROLE OF GALACTOFURANOSE IN VIRULENCE

Galactose is a hexose and a C-4 epimer of glucose (Fig. 1). In mammals, galactose exists only in the pyranoside form (Galp) linked to other carbohydrates as an essential component of glycolipids and glycoproteins [29]. The main role of galactose in humans comes from consumption of dairy products and its metabolism occurs through the Leloir or Isselbacher pathways [30-31]. Galactose in the furanoside (Galf) form is not found in mammals; however, Galf is an important building block of glycans of the cell wall and cell surface in several pathogenic organisms and, therefore, its biosynthesis is a strategic target in the discovery of anti-microbial treatments [14]. The specific role of Galf-containing molecules in *Leishmania spp.*, *T. cruzi*, and *A. fumigatus* is described in this section.

Fig. (1). Structures of β-D-galactopyranose (A) and β-D-galactofuranose (B).

#### 2.1. Galactofuranose in *T. cruzi*

In *T. cruzi*, β-Galf is found in glycoinositolphospholipids (GIPLs) and glycosylphosphatidylinositol (GPI) anchor proteins [32-33]. These glycoconjugates are highly expressed throughout the life cycle of *T. cruzi* and are the main component of the parasite dense surface coat, which has a protective function in parasite survival in the hydrolytic and digestive environment inside their hosts and are important for proliferation [34-36]. For instance, a 45 kDa GPI-mucin is expressed only in invasive trypanastigotes and not in non-invasive amastigotes [37]. Using specific monoclonal antibodies against this protein prevented adhesion of *T. cruzi* to heart myoblasts [37]. These results suggest that Galf-containing glycoconjugates are involved in the mechanism of myocardial invasion by *T. cruzi*.

#### 2.2. Galactofuranose in *Leishmania spp.*

In *L. major*, Galf is found in the oligosaccharide core of lipo-phosphoglycans (LPG) and glycoinositolphospholipids (GIPL) that are essential for parasite survival in the midgut of the vector insect and for parasite transmission to the mammalian host [38-40]. GIPL-1 from *L. major* has been shown to contribute to the infection process [41-42]. LPG deletion mutants in *L. major* showed LPG involvement in resistance to oxidative stress and evasion of the human immune system [39-40].

#### 2.3. Galactofuranose in *A. fumigatus*

Of the vast *Aspergillus* genus that includes over 185 species, *A. fumigatus* and *A. niger* are among the ~20 reported human fungal pathogens that cause a variety of opportunistic diseases facilitated by the suppression of the immune system [43]. Galf has been identified in both organisms and is an important component in the fungal cell wall assembly, where it was found in galactomannan, glycoproteins, sphingolipids, and lipid-linked glycans [44-48]. In *A. fumigatus*, Galf accounts for up to 5% of the dry weight, and is important for fungal growth and cell wall biosynthesis, cell morphogenesis and wall architecture, hyphal adhesion, spor development, and pathogenesis [22-23, 49].

### 3. UDP-GALACTOPYRANOSE MUTASE: AN ATTRACTIVE DRUG TARGET AGAINST EUKARYOTIC HUMAN PATHOGENS

UDP-Galactopyranose mutase (UGM) is a flavin-dependent enzyme that catalyses the isomerization of UDP-Galp to UDP-Galf through a unique type of flavin-dependent catalysis (Fig. 2) [13, 50-52]. The gene encoding for UGM (gflf) was first identified in prokaryotes in 1996 while studying the *Escherichia coli K12* O antigen [53]. In the following years, it was identified in other pathogens including the eukaryotes *L. major*, *T. cruzi*, and *A. fumigatus* [54]. Deletion of the UGM gene leads to attenuated virulence in *L. major* [55]. In *T. cruzi*, the role of Galf in binding to mammalian cells has been shown; however, deletion of the UGM gene in this parasite have not been performed.

Deletion of the UGM gene in *A. fumigatus*, in addition to causing attenuated virulence, leads to cell-wall morphology defects, increased sensitivity to anti-fungal drugs, and growth reduction [21-22]. These results validate UGM as a potential target for the development of drugs against these eukaryotic pathogens.

#### 3.1. Primary Structure of UGMs

The polypeptide chain lengths of eukaryotic UGMs are generally about 100 amino acid residues longer than those of the prokaryotic enzymes (Fig. 3). Sequence alignment reveals a moderate identity (47-60%) among the eukaryotic UGMs from *A. fumigatus* (AfUGM), *L. major* (LmUGM), and *T. cruzi* (TcUGM), and a slightly lower (37-44%) sequence identity among the prokaryotic homologs from *Escherichia coli* (EcUGM), *Mycobacterium tuberculosis* (MtUGM), *Klebsiella pneumoniae* (KpUGM), and *Deinococcus radiodurans* (DrUGM). However, the sequence identity between eukaryotic and prokaryotic UGM groups is surprisingly low (14-18%) (Table 1). Conserved among all UGMs is the GxGxxG motif that is necessary for FAD binding. Only partial conservation of active site residues is observed (Fig. 3). The low amino acid conservation and the extra amino acid sequence in eukaryotic UGMs endows these enzymes with unique structural features that are important for enzyme function; these are discussed in the next section. Interestingly, an obvious NAD(P)H binding domain or motif is not found in this family of enzymes. This is intriguing since this class of enzymes has been shown to function only in the reduced state.

#### 3.2. 3-Dimensional Structure of UGMs

Whereas several crystal structures of bacterial UGMs have been determined [56-58], among the eukaryotic enzymes, only the structure of *A*. *UGM* is known at this time (Table 2) [59-60]. A*UGM* is a mixed α/β fold protein containing three structural domains (Fig. 4). Domain 1 includes a Rossmann fold core and participates in FAD binding. Domains 2 and 3 function in substrate binding [59]. Interestingly, an obvious NAD(P)H binding domain or motif is not found in this family of enzymes. This is summarized below.

The conformations of the flavin and flavin-protein interactions are highly conserved between prokaryotic and eukaryotic UGMs (Table 3). The NADP+ xG motif of the oxidized enzyme is planar, which is typical for flavoenzymes. Characterization of the reduced FAD conformation is important for understanding the chemical mechanism because the reduced FAD functions as a nucleophile in the UGM reaction. This function places certain structural restrictions on the flavin isoalloxazine. In particular, steric considerations suggest that the reduced isoalloxazine should be nonplanar with the wings of the isoalloxazine bending away from the substrate. Indeed such a conformation is observed in reduced *A*. UGM and DrUGM.
In contrast to bacterial UGMs, in bound UDP. In tated by 110º. There is also a variation in the conformation of p Arg and Tyr residues are conserved and participate in electrostatic hydrogen bonding, and the anomeric carbon of the sugar is placed about 90º with respect to bacterial.

The isoalloxazine described above.

Comparison of the structures of β-UGM and bacterial UGMs with UDP-Galp reveals conserved motifs and important differences. In all the complex structures (Tab. 2 and Tab. 3), the OH-4 of the Galp moiety interacts with the flavin O-4 through hydrogen bonding, and the anomeric carbon of the sugar is placed within a short distance from the flavin N-5 (Fig. 5). Also, several Arg and Tyr residues are conserved and participate in electrostatic interactions with the pyrophosphate portion of UDP-Galp (Tab. 3). In contrast to bacterial UGMs, in β-UGM the OH-6 of Galp is rotated by 110º. There is also a variation in the conformation of bound UDP. In β-UGM, UDP is displaced by ~4 Å and rotated by about 90º with respect to bacterial KpUGM and DrUGM. This allows for the hydrogen bonding of uracil with Gln107, a residue that is not present in bacterial UGMs. These structural differences in substrate recognition between bacterial and eukaryotic UGMs could have implications for inhibitor discovery. In particular, it seems unlikely that compounds that target the uridine site of bacterial UGMs will be effective against eukaryotic UGMs.

Large protein conformational changes (>10 Å movements) accompany substrate binding in UGMs. β-UGM, and presumably other eukaryotic UGMs, exhibit larger conformational changes. Comparison of the structures of the substrate-free and substrate-bound forms revealed two flaps (residues 179–187 and 203–209) that close down over the substrate like the flaps of a box top (Fig. 5). Whereas the 180s flap is analogous to the mobile loop of bacterial UGMs, the 200s flap is unique to eukaryotic UGMs. The dramatic closing of the active site in bacterial and eukaryotic UGMs is an important aspect of the catalytic mechanism. These movements result in the assembly of the constellation of residues that position the substrate for nucleophilic attack by the FAD. Furthermore, the closing of the active site prevents diffusion of intermediates, such as UDP, out of the active site during the catalytic cycle.

Various oligomeric states have been observed for UGMs in solution. The oligomeric states of several UGMs have been determined from size exclusion chromatography, small-angle X-ray scattering (SAXS), and analysis of protein-protein interfaces in crystal lattices (Tab. 4). Bacterial UGMs tend to form dimers in solution. EcUGM, KpUGM, and MtUGM form a semicircular dimer [58, 63]. The fact that this structure is formed by multiple UGMs in different crystal lattices attests to its veracity. The oligomeric state of DrUGM is less certain. The classic UGM semicircular dimer is not found in the DrUGM lattice, and solution studies of the oligomeric state have not been performed on the enzyme. The DrUGM crystal lattice implies decameric and dimeric assemblies, but clearly additional work is needed to determine the oligomeric state and quaternary structure of DrUGM. In contrast, the oligomeric state of DrUGM has been unequivocally determined using a combination of SAXS and X-ray crystallography [59]. These studies have shown that AβUGM is unique among UGMs in that it forms a tetramer in solution [59, 64]. The AβUGM tetramer is a dimer-of-dimers assembly (Fig. 6). Unique structural features of AβUGM that are absent in the bacterial enzymes enable tetramerization. These extra elements include a longer C-terminus, an extra helix in domain 2, and extension of another helix of domain 2 (Fig. 4).

Because the reduced FAD is essential for catalysis, the mechanism by which the enzyme is activated by flavin reduction is an important aspect of UGM biochemistry. Insight into the structural underpinnings of this mechanism has been obtained by comparing crystal structures of oxidized and reduced UGMs. Inspection of the bacterial enzyme structures reveals little difference between the oxidized and reduced conformations, aside from the bending of the isoalloxazine described above.
Fig. (3). Sequence alignment of eukaryotic (group 1) and prokaryotic (group 2) UGMs. The residues conserved in all of the sequences are shown in red shaded boxes. Those conserved only in one group are shown in red color. Active site residues are marked with a star, and those interacting with flavin are marked with β-sheets (arrows; TT – strict turns; TL – loose turns). The α-helix (spiral) and β-sheets of the UGMs are depicted on top. ClustalW was used to generate the alignment and ESPript 2.2 to create the figure. (The color version of the figure is available in the electronic copy of the article).
Table 2. Available UGM Crystal Structures in the Protein Data Bank (PDB)

| UGM       | Active site ligand | PDB code [94] | Ref. |
|-----------|--------------------|---------------|------|
| AfUGM<sub>red</sub> | UDP-Galp          | 3UTF [59]     |      |
| A<sub>f</sub>UGM<sub>red</sub> | UDP              | 3UTG [59]     |      |
| A<sub>f</sub>UGM<sub>red</sub> | UDP-Galp          | 3UTH [59]     |      |
| A<sub>f</sub>UGM<sub>ox</sub> | sulfate          | 3UTE [59]     |      |
| A<sub>f</sub>UGM<sub>red</sub> | UDP-Galp          | 3UKF [60]     |      |
| A<sub>f</sub>UGM<sub>red</sub> | UDP-Galp          | 3UKH [60]     |      |
| A<sub>f</sub>UGM<sub>ox</sub> | UDP              | 3UKL [60]     |      |
| EcUGM<sub>ox</sub> | FMN               | 3KYB n.a.*    |      |
| KpUGM<sub>ox</sub> | UMP, UDP-Glp      | 3GF4 [61]     |      |
| KpUGM<sub>ox</sub> | UDP-Galp          | 3INR [62]     |      |
| KpUGM<sub>red</sub> | UDP, UDP-Galp     | 3INT [62]     |      |
| KpUGM<sub>red</sub> | UDP-Galp          | 1WAM [58]     |      |
| KpUGM<sub>ox</sub> | UDP              | 2BI7 [58]     |      |
| KpUGM<sub>red</sub> | UDP              | 2BI8 [58]     |      |
| MtUGM<sub>ox</sub> | UDP              | 1V0J [58]     |      |
| DrUGM<sub>ox</sub> | UDP              | 3HE3 [56]     |      |
| DrUGM<sub>ox</sub> | UDP-Galp          | 3HDY [56]     |      |
| DrUGM<sub>ox</sub> | UDP-Galp          | 3HDQ [56]     |      |
| DrUGM<sub>ox</sub> | UDP, UDP-CH<sub>2</sub>-Galp | 3MJ4 [95] |      |

*Gruber TD, Dimond MC, Kiesling LL, Forest KT, Structure of UDP-galactopyranose mutase bound to flavin mononucleotide. Unpublished results.

Table 3. Ligand Interactions with UGMs

| Type of interaction                     | A<sub>f</sub>UGM<sub>red</sub> | EcUGM<sub>ox</sub> | KpUGM<sub>red</sub> | DrUGM<sub>red</sub> |
|----------------------------------------|---------------------------------|-------------------|-------------------|-------------------|
| UDP-Galp contacts                      |                                 |                   |                   |                   |
| π-π stacking with uracil               | Y104, F158                      | n.a.*             | F152, Y155        | F176, Y179        |
| H-bonding with uracil                 | F106, Q107                      |                   | N270              | F175, N296        |
| Interactions with diphosphate          | R182, Y317, Y419                 |                   | R174, Y185, R280, Y314 | R198, Y209, R305, Y335, Y370 |
| H-bonding with Galp                   | R182, N207, N457                | R84, Y349         |                   | H109, R305        |
| Other important amino acids for        | N163, W167                       |                   | W160              | T180, W184        |
| substrate binding                      |                                 |                   |                   |                   |
| FAD contacts                           |                                 |                   |                   |                   |
| H-bonding with ribose                 | H63, G456, S461                 | N39, Y347         | H60, L350, T355   | H85, Y371         |
| Interaction with pyrophosphate        | T18, L46, R447                  |                   | F12, N39, R340    | F13, S14, N41, R343 |
| π-π stacking with isoxaloxazine        | H63                             | H56               | H60               | H85               |
(Table 3) Contd…..

| Type of interaction | AfUGMred | EcUGMred | KpUGMred | DrUGMred |
|---------------------|----------|----------|----------|----------|
| Interaction with adenine | D38, S39, V242 | E31, K32, D212, F213 | F219 | R60, D242, Y243 |
| H-bonding with isoalloxazine | V64, Q458 | I57, M349, Y346 | I61, M352 | I86, M373 |
| H-bonding with N5 | G62 | A55 | P59 | P84 |
| H-bonding with ribose | D38 | E31 | | |

FAD-substrate contacts

OH-4(Galp) and CO-4(FAD) n.a.* OH-4(Galp) and CO-4(FAD) OH-4(Galp) and CO-4(FAD)

*Not available.

Table 4. Amino Acid Composition and Molecular Weight of UGMs from Different Organisms

| Organism         | Oligomeric state in solution | Number of amino acids | MW of monomer, Da | Ref. |
|------------------|-----------------------------|-----------------------|-------------------|------|
| A. fumigatus     | Tetramer*<sup>a</sup>        | 510                   | 56,820            | [59, 64] |
| T. cruzi         | Monomer*<sup>b</sup>        | 480                   | 54,690            | [73] |
| L. major         | Monomer*<sup>c</sup>        | 491                   | 54,970            | [96] |
| E. coli          | Dimer*<sup>d</sup>          | 367                   | 42,970            | [63, 97] |
| K. pneumoniae    | Dimer*<sup>d</sup>          | 384                   | 44,460            | [58, 61-62] |
| M. tuberculosis  | Dimer*<sup>d</sup>          | 399                   | 45,820            | [58] |
| D. radiodurans   | Not determined              | 397                   | 45,700            | [56, 98] |

*<sup>a</sup>Determined by size exclusion chromatography.
*<sup>b</sup>Determined by SAXS.
*<sup>c</sup>Determined by light scattering.
*<sup>d</sup>Inferred from analysis of protein-protein interfaces in the crystal lattice.

Fig. (4). Protomer structure of reduced AfUGM complexed with UDP-Galp. FADH<sup>+</sup> and UDP-Galp are colored gray and green, respectively. The flexible active site flaps are colored red. (The color version of the figure is available in the electronic copy of the article).
Initial results for A½UGM potentially reveal a much more complex mechanism for activating eukaryotic UGMs (Fig. 7). Two crystal forms of A½UGM have been described, a P6522 form reported by us [59], and a P1 form reported by Sanders’ group [60]. Although the interpretation of these structures is complicated by the binding of sulfate ion in the P6522 form, and weak electron density in the P1 form, the structures tantalizingly imply large conformational changes involving the conserved histidine loop (G61-G62-H63). The structures show that, in the oxidized enzyme, conserved His63 is near the pyrimidine ring of the isoalloxazine and the carbonyl of Gly62 points away from the isoalloxazine (Fig. 7A and 7B), which is unprecedented for UGMs. The structures further indicate that flavin reduction induces a crankshaft rotation of the loop backbone, which reverses the orientation of the Gly62 carbonyl bond vector and moves the imidazole of His63 by over 5 Å. These changes bring the carbonyl of Gly62 within hydrogen bonding distance of the N5 of the reduced flavin and move His63 to the si face of the isoalloxazine where it stacks in parallel with the isoalloxazine and forms a hydrogen bond with the OH-2’ of the ribityl (Fig. 7C). These interactions between the histidine loop and the flavin...
help stabilize the reduced state of the enzyme and are found in all other UGM structures. The presence of two Gly residues in the loop is unique to eukaryotic UGMs and probably accounts for the large conformational changes seen in AfUGM compared to the bacterial enzymes. More research is needed to validate these conformational changes for AfUGM and determine whether other eukaryotic UGMs exhibit analogous movements.

### 3.3. Chemical Mechanism of Eukaryotic UGMs

Despite structural differences, the unique chemical mechanism utilized by UGMs is conserved among the members of this enzyme family. For all UGMs, only the reduced form of the enzyme is active [63], although the reaction does not involve a net gain or loss of electrons, which is common among many other classes of FAD-dependent enzymes [65-67]. The reported steady-state values with UDP-Gal as substrate and dithionite as the reductant show only minor differences in $k_{cat}$ and $k_{cat}/K_M$ among members of the UGM family (Table 5).

The enzymatic reaction has been shown to involve cleavage of an anomeric bond and the formation of a Galp-FAD adduct (Fig. 8) [68]. This process was initially thought to involve one of three mechanisms: a single-electron transfer from the reduced flavin to a postulated oxocarbenium intermediate of Galp [69-70] or a nucleophilic substitution via an S_N1 or S_N2 mechanism, both leading to the formation of a Galp-FAD adduct [71-72]. While the Galp is bound to the FAD it undergoes ring opening and closing rearrangement and, after nucleophilic attack by UDP, the UDP-Galp is produced. Formation of the FAD-sugar adduct has been demonstrated by chemical quenching, trapping, and characterization by mass spectrometry in both eukaryotic and prokaryotic UGMs [71, 73]. Rapid reaction kinetic analysis with reduced TcUGM and UDP-Galp did not show the presence of a transient flavin semiquinone, inconsistent with a single electron transfer step. Instead, absorbance changes consistent with the formation of a flavin iminium ion, were observed and occur very fast [73]. The structures of AfUGM and prokaryotic UGMs in complex with UDP-Galp clearly show that the substrate binds in a conformation optimal for direct attack by the flavin N5. Furthermore, linear free energy relationship (LFER) studies with prokaryotic UGM, reconstituted with various FAD analogs, show changes in $k_{cat}$ values that correlate linearly with changes in the nucleophilicity of the flavin N5 (slope of $\rho = -2.4 \pm 0.4$), which is consistent with an S_N2 mechanism [72]. Viscosity effect studies showed that product release was not rate limiting in the case of TcUGM [73].

In vivo, all UGMs function in an aerobic environment. Therefore, a system for the generation and maintenance of the reduced

![Fig. (7). Conformations of the histidine loop in AfUGM structures. A, oxidized P622 form (PDB code 3UTE); B, oxidized P1 form (PDB code 3UKH); C, reduced enzyme (PDB code 3UTF).](image-url)

| Organism          | $k_{cat}$, s$^{-1}$ | $K_M$, $\mu$M | $k_{cat}/K_M$, $\mu$M$^{-1}$ s$^{-1}$ | Ref.  |
|-------------------|---------------------|---------------|--------------------------------------|-------|
| *A. fumigatus*    | 72 ± 4$^*$          | 110 ± 15$^*$  | 0.65 ± 0.09$^*$                      | [59, 64] |
| *T. cruzi*        | 13.4 ± 0.3$^*$      | 140 ± 10$^*$  | 0.093 ± 0.006$^*$                    | [73]  |
|                   | 11.5 ± 0.4$^*$      | 200 ± 20$^*$  | 0.056 ± 0.005$^*$                    |       |
|                   | 8.4 ± 0.9$^*$       | 690 ± 150$^*$ | 0.012 ± 0.001$^*$                    |       |
| *L. major*        | 5 ± 0.2$^*$         | 87 ± 11$^*$   | 0.057 ± 0.006$^*$                    | [96]  |
| *E. coli*         | 27$^*$              | 22$^*$        | 1.22$^*$                             | [99]  |
| *K. pneumoniae*   | 5.5 ± 0.7$^*$       | 43 ± 6$^*$    | 0.12 ± 0.02$^*$                      | [100] |
| *M. tuberculosis* | 8$^*$               | 13$^*$        | 0.62$^*$                             | [101] |
| *D. radiodurans*  | 66 ± 2.4$^*$        | 55 ± 7$^*$    | 1.18$^*$                             | [56]  |

$^*$Reduced with 5-20 mM dithionite. $^1$Reduced with 0.5 mM NADPH. $^2$Reduced with 2.5 mM NADH.
UGM, the bacterial enzymes are not effective NAD(P)H oxidases. binds with micromolar affinity, it is clear that relative to eukaryotic minutes [74]. Taking into account that NADPH is capable of reduc-
Kp
zymes [73]. It has been previously reported that the activity of contrast, MtUGM is unable to effectively react with reduced coen-
Mt
UGM is unable to effectively react with reduced coen-
Tc
UGM was enhanced by the addition of NADH or NADPH [74-75]. The binding affinities or rates of reduction were not reported, however, the rate enhancement was observed at concentrations greater than 20 mM NADH and at incubation times longer than 10 minutes [74]. Taking into account that NADPH is capable of reduc-
Tc
UGM with a rate constant in the second time scale and it
Tc
UGM with a rate constant in the second time scale and it

Substrate | \( k_{\text{red}} \) (s\(^{-1}\)) | \( K_d \) (\( \mu M \)) | \( k_{\text{red}}/K_d \) (\( \mu M^{-1} \) s\(^{-1}\))
---|---|---|---
NADH | 0.085 ± 0.0006 | 550 ± 10 | 0.00015 ± 0.000002
NADPH | 0.600 ± 0.006 | 98 ± 3 | 0.0061 ± 0.0001

The mechanism shown in (Fig. 8) was recently proposed for TcUGM [73]. Although, as mentioned above, the initial steps in the catalysis, NAD(P)H binding and subsequent FAD reduction, occur much less effective in prokaryotic UGMs, the steps leading to the conversion of Galf are conserved in these enzymes [71, 73].

4. METHODS FOR HIGH THROUGHPUT SCREENING FOR UGM INHIBITORS

With the exponential advance of robotics, data collection, and analysis methods, high throughput screening (HTS) provides an effective and relatively fast preliminary analysis of chemical libraries composed of thousands of chemical compounds for the search of potential chemotherapeutics [76-80]. Whether the goal is to find an effective inhibitor for a well-explored enzyme or to match existing drugs to new macromolecular targets, HTS provides the rational starting point in the drug discovery process. Elimination of ineffective drug candidates early on using HTS is essential and saves time and resources during later stages of drug development, since libraries can contain thousands of compounds with a 0.1-0.2% probability of identifying positive hits [81-82]. Thus, the development of a successful assay for HTS is extremely important.

Standard methods used to assay UGMs include: HPLC analysis, UV/Vis and stopped-flow spectroscopy, redox potentiometry, fluorescence polarization, and radiochemical detection [64, 69, 71, 83-84]. The HPLC method has been adopted by many groups, as it easily allows one to measure the activity of UGMs both qualitatively and quantitatively. In general, the assay monitors the reverse reaction, UDP-GalF to UDP-Gal conversion. Both the substrate and the product are easily detected at 262 nm, which corresponds to the absorbance maxima of UDP. Despite the broad utilization of the HPLC method by many research groups, this assay is not suitable for screening large chemical libraries because of the lengthy HPLC run times and because it is not suitable for running multiple measurements at once. A radioactive assay based on the generation and monitoring of tritiated formaldehyde, from the radioactive UDP-GalF degradation product, was used in the screening of 1,300 potential inhibitors against prokaryotic MtUGM. However, the poor sensitivitiy of the assay due to the equilibrium of the reaction not favoring the formation of UDP-GalF isomer was an essential drawback of this approach for high throughput screening applications [84]. Other reported assays used in HTS against UGMs are based on fluorescence polarization (FP) [83, 85-86]. FP relies on changes in the tumbling of a chromophore as it transitions from the enzyme-bound to the free-state due to competition by an inhibitor. This assay is simple, fast, and can be done on a small scale. Various fluorescent probes based on UDP were synthesized to develop a FP assay for both prokaryotic and eukaryotic UGMs (Fig. 9). Chromophore 1 was shown to effectively bind prokaryotic UGMs from M. tuberculosis and K. pneumoniae and used in HTS with the library strategically derivatized from a thiazolidinone core [85]. Contrary to prokaryotic UGMs, the fluorescein fluorophore was not as effective and specifically derivatized from a thiazolidinone core [85]. Contrary to prokaryotic UGMs, the fluorescein fluorophore was not as effective and specifically derivatized from a thiazolidinone core [85]. Contrary to prokaryotic UGMs, the fluorescein fluorophore was not as effective and specifically derivatized from a thiazolidinone core [85]. Contrary to prokaryotic UGMs, the fluorescein fluorophore was not as effective and specifically derivatized from a thiazolidinone core [85].
served, inhibitors of AfUGM might also be effective against the other eukaryotic UGM homologs.

5. IN SILICO DRUG DESIGN AND ITS APPLICATIONS TO UGM

Protein crystal structures serve as the blueprints for computer-guided molecular recognition, design, and virtual-screening of drug-like molecules and diagnostic probes [87]. There are three extensively used strategies for in silico drug design: de novo design, fragment-based drug discovery [88-89], and virtual screening [90]. The first two are very similar in their algorithms and concepts: both are based on design “from scratch”, involving the screening of small pharmacophoric chemical blocks (or fragments) within the three-dimensional active site of the target enzyme. At later stages of the experiment, these fragments are further expanded (“grown”) upon other moieties or directly joined together through a chemical bond or a linker. Virtual screening deals with the vast libraries of small chemical compounds utilizing high-throughput docking and pharmacophore-based searching algorithms and can be classified into two broad categories: ligand-based or structure-based docking and scoring [90-91].

Recently, a virtual screening using various computational tools toward the identification of inhibitors against EcUGM (also KpUGM and MtUGM) was applied to a small-molecule library comprised of 84,000 compounds (LeadQuest, Tripos, Inc.) [57]. A total of 13 compounds (0.015% of the library) were identified as positive hits and tested for inhibitory activity toward KpUGM and MtUGM. Only three compounds were shown to be effective inhibitors and had comparable affinity with the best previously published prokaryotic UGM inhibitors (IC₅₀ 7.2 - 62 μM, [84-86, 92-93]). The effective application of in silico screening to bacterial UGMs suggests that a similar approach can be applied to eukaryotic enzymes. Furthermore, it is expected that an in silico screening approach with the structure of AfUGM will also identify potential inhibitors for TcUGM and LnUGM.

6. CONCLUDING REMARKS

Galf is important for cell wall biosynthesis and cell surface glycan structures in bacteria, fungi, and parasites. Galf is either essential for growth or important for pathogenesis, making enzymes in its biosynthetic pathway potential drug targets. In this pathway, UGM is an ideal target for drug discovery because this enzyme is absent in humans, and its structure and chemical mechanism are unique. During the past decade, the catalytic mechanism was fully elucidated and the structural differences between prokaryotic and eukaryotic UGM characterized. Recent discoveries in the field of eukaryotic UGMs set the stage for the identification of inhibitors that might lead to drugs for the treatment of neglected diseases like Chagas disease, leishmaniasis, and fungal infections caused by Aspergillus spp. In principle, every conformation along the catalytic cycle is a potential design target, including both the active, reduced enzyme and the inactive, oxidized one. Strategies toward developing effective drugs can include the design of a small-molecule competitive inhibitor with much higher binding affinity to UGM with respect to UDP-Galp₂f, or even molecules that do not bind to the active site but interact with the mobile loops to prevent proper binding of the substrate.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

UDP = Uridine diphosphate
UGM = UDP-galactopyranose mutase
Galp = Galactopyranose
Galf = Galactofuranose
WHO = World Health Organization
NTD = Neglected Tropical Diseases
A. fumigatus = Aspergillus fumigatus
A. niger = Aspergillus niger
T. cruzi = Trypanosoma cruzi
L. major = Leishmania major
Leishmania spp. = Leishmania species
ABPA = Allergic bronchopulmonary aspergillosis
IPA = Invasive pulmonary aspergillosis
AIDS = Acquired Immune Deficiency Syndrome
ICU = Intensive care unit
GIPLs = Glycoinositolphospholipids
GPI = Glycosylphosphatidylinositol
FAD = Flavin adenine dinucleotide
NAD(P)H = Nicotinamide adenine dinucleotide (phosphate)
SAXS = Small-angle X-ray scattering
LFER = Linear free energy relationship
HTS = High throughput screening
HPLC = High performance liquid chromatography
UV/Vis = Ultraviolet/visible
FP = Fluorescence polarization
TAMRA = Tetramethylrhodamine

Fig. (9). Fluorescent probes used in HTS assays for the identification of UGM inhibitors.
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