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Identification of eukaryotic UDP-galactopyranose mutase inhibitors using the ThermoFAD assay

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ABSTRACT

Aspergillus fumigatus is a human pathogen responsible for deadly infections in immune-compromised patients. A potential strategy for treating *A. fumigatus* infections is by targeting the biosynthesis of cell wall components, such as galactofuranase, which is absent in humans. Galactofuranose biosynthesis is initiated by the flavoenzyme UDP-galactopyranose mutase (UGM), which converts UDP-galactopyranose (UDP-Gal*p*) to UDP-galactofuranose (UDP-Gal*f*). UGM requires the reduced form of the flavin for activity, which is obtained by reacting with NADPH. We aimed to identify inhibitors of UGM by screening a kinase inhibitor library using ThermoFAD, a flavin fluorescence thermal shift assay. The screening assay identified flavopiridol as a compound that increased the melting temperature of *A. fumigatus* UGM. Further characterization showed that flavopiridol is a non-competitive inhibitor of UGM and docking studies suggest that it binds in the active site. This compound does not inhibit the prokaryotic UGM from *Mycobacteria tuberculosis*.

1. Introduction

Aspergillus fumigatus is a common, opportunistic human pathogen responsible for allergic bronchopulmonary aspergillosis and invasive pulmonary aspergillosis. The mortality rate among immunocompromised patients infected with *A. fumigatus* is >50% [1]. Targeting enzymes that play key roles in cell wall biogenesis has proven to be an effective approach for the development of antifungal drugs because the fungal cell wall plays a crucial role in host-pathogen interactions and is essential for maintaining cell integrity [2]. The *A. fumigatus* cell wall consists of an arrangement of polysaccharides, composed mainly of branched β-1,3-glucans cross-linked to chitin with an external core of glucan and galactomannan [3]. The galactomannan core is the major antigen produced during infection [4] and is constructed of branched mannose containing galactofuranose (Gal*f*) and/or galactopyranose (Gal*p*) moieties linked linearly in side chains terminated with Gal*f* non-reducing end units [4–6]. Gal*f* is a five-member cyclic hexose found in several pathogens but absent in humans [7]. The synthesis of Gal*f* starts in the cytosol where UDP-Gal*p* is transformed to UDP-Gal*f* by the flavoenzyme UDP-Gal*p* mutase (UMG, Fig. 1). UGM is essential for pathogenesis in *Mycobacterium tuberculosis* [8] and larva hatching and motility in the nematodes *Brugia malayi* and *Caenorhabditis elegans* [9–11]. Deletion of the UGM gene in *A. fumigatus* results in depletion of Gal*f*, reduction in cell wall thickness, and attenuation of virulence in mice models [12]. UGM catalyzes a non-redox reaction; however, the flavin is required to be in the reduced form [13] for activity, with the flavin reduction occurring by reaction with NADPH [14].

Here, we present the successful implementation of a fluorescence thermal shift assay known as ThermoFAD to screen for inhibitors of *A. fumigatus* UGM (AfUGM). This assay monitors the fluorescence of the FAD bound to AfUGM during thermal denaturation experiments to calculate the melting temperature (T_m) [15,16]. Increase of the T_m value in the presence of small molecule suggests that the compound is binding to the protein. Using this assay, a kinase inhibitor library was screened against AfUGM and flavopiridol was identified as a potential inhibitor. Addition of this compound to a solution of AfUGM resulted in an increase in the melting temperature, suggesting that a protein-ligand complex with higher stability is formed. Isothermal titration calorimetry experiments confirmed the complex formation with a K_D value of 38 μM. Inhibition studies monitoring the mutase activity showed that flavopiridol functions as a non-competitive inhibitor. Docking

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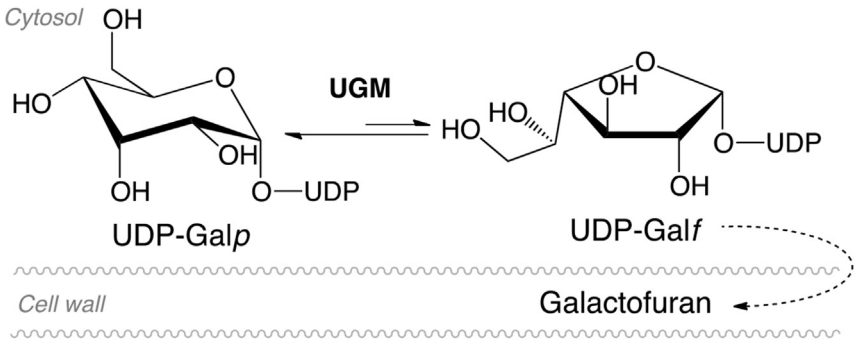

Fig. 1. The reaction catalyzed by AfUGM. This reaction is favored in the direction of UDP-Gal*p* synthesis.

studies suggest that flavopiridol binds in the active site. Flavopiridol, also known as Alvocidib, is an inhibitor of the CDK kinases and is currently under clinical trials for the treatment of acute myeloid leukemia.

2. Material and methods

2.1. Materials

Buffers, antibiotics and bacterial growth media were obtained from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich (St. Louis, MO). Turbo BL21 (DE3) chemically competent cells were obtained from Genlantis (San Diego, CA) and pVP55A vector was obtained from the Center for Eukaryotic Structural Genomics at the University of Wisconsin-Madison [17]. For protein purification, immobilized metal affinity chromatography (IMAC) was used in an AKTA prime system from GE Healthcare (Chicago, IL). Acquity ultra-performance liquid chromatography (UPLC) and Amide (1.7 μm, 2.1 mm × 100 mm) analytical columns were obtained from Waters Co. (Milford MA). NADPH was obtained from EMD Biosciences (Billerica, MA). UDP-Gal*f* was synthesized as described previously [13]. Kinase Inhibitor Library (L1200) was obtained from Selleckchem (Houston, TX). The fluorescence thermal shift assay was performed in an RT-PCR (Applied Biosystems 7300) using 96-well RT-PCR plates (Microamp 4306737) and optical adhesive films (MicroAmp 431197971). Isothermal titration calorimetry (ITC) measurements were performed in Auto-ITC 200 from Malvern Instruments (Alvern, UK) and analyzed using the Microcal Origin version 7.0 (OriginLab). Flavopiriol was purchased from ApexBio (Houston, TX).

2.2. Expression and purification of A. fumigatus UGM and M. tuberculosis UGM

AfUGM was expressed in the vector pVP55A as previously reported [13]. Briefly, 6-L of terrific-broth (TB) auto induction media containing 100 μg/mL ampicillin were inoculated with 8 mL of overnight culture of BL21 Turbo cells transformed with the vector pPV55A and incubated at 37 °C until an optical density at 600 nm (OD_{600}) value of 3 was reached, at which point the temperature was dropped to 18 °C and the cultures were incubated for 18 additional hours. Cells were harvested by centrifugation at 5000x*g* for 20 min at 4 °C. The final wet-cell pellet (75 g) was stored at −80 °C. For protein purification, the cell paste was resuspended in 250 mL of buffer A (25 mM HEPES, 300 mM NaCl, 25 mM imidazole, pH 7.5) and incubated with 25 mg/mL of lysozyme, DNAse I, and RNAse for 45 min at 4 °C with constant stirring. The resulting solution was sonicated in an ice bath for 15 min at 70% amplitude with cycles of 5 s on and 10 s off. The lysate was centrifuged at 45,000 *g* for 45 min and the supernatant was collected and loaded onto three in-tandem 5-mL HisTrap columns previously equilibrated with buffer A. After washing the column with 100 mL of buffer A, AfUGM was eluted with 100 mL of Buffer B (25 mM HEPES, 300 mM NaCl, 300 mM imidazole, pH 7.5). The yellow fractions that contained AfUGM were pooled. To remove the 8xHis-tag, 8xHis-tobacco etch virus (8xHis-Tev) protease (1:20 ratio) was added and the solution was dialyzed at 4 °C with constant stirring in buffer C (25 mM HEPES, 300 mM NaCl, pH 7.5). The next day, the solution was loaded onto an IMAC previously equilibrated with buffer C, and the flow-through containing AfUGM was collected and dialyzed against 25 mM HEPES with 100 mM NaCl. Protein was flash frozen and stored at -80 °C. *M. tuberculosis* UGM (MtUGM) was purified as described previously [13].

2.3. High-throughput screening using the ThermoFAD assay

High-throughput screening (HTS) of the Kinase Inhibitor Library were performed at the Virginia Tech Center for Drug Discovery Screening Laboratory. For the screening, 96 well RT-PCR plates were used (Microamp 4306737). The AfUGM concentration was 1 mg/mL and 200 μM of the compounds were added, yielding a final DMSO concentration of 2% in a total volume of 20 μL of 50 mM potassium phosphate buffer, pH 7.0. The melting temperature of AfUGM was determined by measuring flavin fluorescence as a function of temperature (20-90 °C, 1 °C step), using an excitation wavelength range between 470 and 500 nm and a fluorescence emission between 523 and 543 nm [16]. AfUGM with 2% DMSO was used as the negative control (no change in T_m) and AfUGM with 10 mM UDP as the positive control (change in T_m). The Z prime (Z′) value was determined using equation (1) where $\sigma_{N,P}$ and $\mu_{N,P}$ represent the standard deviation and the mean value of the negative and positive controls, respectively.

$$Z' = 1 - \frac{3(\sigma_N + \sigma_P)}{|\mu_N - \mu_P|} \quad (1)$$

2.4. Activity assay, IC_{50} and K_i

The activity of AfUGM and IC_{50} was determined by monitoring the formation of UDP-Gal*p* from UDP-Gal*f* by UPLC. A 30 μL reaction containing 20 μM UDP-Gal*f*, 10 mM sodium dithionite, 10 nM AfUGM (concentration based on FAD), 2% DMSO, buffer (25 mM HEPES containing 125 mM NaCl) and flavopiridol (0-1 mM) was incubated at 25 °C for 3 min. Protein and inhibitor were pre-incubated for 5 min at 25 °C and the reaction was started with the addition of 3 μL of 200 μM UDP-Gal*f*. The reaction was quenched with the addition of 90 μL acetonitrile. After removing

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the protein by centrifugation, 5 μL of sample was injected into an Amide column (Acquity UPLC BEH Amide, 1.7 μm, 2.1 mm × 100 mm) at 45 °C. The UPLC column was pre-equilibrated with 75% acetonitrile and 25% of 27 mM aqueous potassium phosphate (pH 4.5). Samples were run at a flow rate of 0.5 mL/min for 5 min. Determination of the inhibition constant (K_i) was performed in the above-mentioned buffer with 10 nM UGM, 10 mM sodium dithionite, and 0, 250, and 500 μM flavopiridol. The concentration of UDP-Gal*f* was varied between 0 and 500 μM and product formation was determined by measuring the absorbance at 260 nm ($\varepsilon_{260} = 10\ mM^{-1}cm^{-1}$) using the UPLC-method, which allows for the simultaneous quantification of substrate and product.

2.5. Isothermal titration calorimetry (ITC) measurements

Binding of small molecules to AfUGM was measured with a MicroAuto-ITC 200 instrument. AfUGM was dialyzed in 50 mM potassium phosphate buffer, pH 7.0, for 2 h, at 4 °C. In all cases, ligands were dissolved in dialysis buffer and protein and ligand were filtered with a 0.2 μm membrane prior to loading. AfUGM concentration in the cell was 46 μM for the binding measurements of $NADP^+$ (16.8 mM in syringe) and UDP (400 μM in syringe). For binding of UDP-Gal*p*, 70 μM UGM was used (in cell) with 5 mM substrate in the syringe. In the case of flavopiridol, 30 μM ligand in the cell was used with or without sodium dithionite (5 mM) and 350 μM AfUGM in the syringe with or without sodium dithionite (5 mM). Titration was performed with 16 injections of 2.5 μL having a spacing of 180 s at 25 °C with continuous stirring at 750 rpm. Thermodynamic parameters were determined from ITC results using Microcal Origin version 7.0 (OriginLab) with the ITC custom add-on installed.

2.6. Data analysis

ThermoFAD assay data were analyzed using the published procedures [18] and GraphPad Prism 6 software. IC_{50} was calculated with KeleidaGraph software.

2.7. Docking studies

The docking studies were conducted in Autodock Vina [19] using the PDB code 3UTF corresponding to the structure of AfUGM in the reduced state [14]. Prior to docking calculations, water molecules were removed from the crystal structure leaving the FAD. The structure of the receptor and the ligand, was prepared using Dock Prep [20]. A grid box centered in the receptor was set large enough to cover the entire surface of a monomer of AfUGM.

3. Results

3.1. High throughput screening

ThermoFAD is a differential scanning fluorimetry assay for flavoenzymes that measures the fluorescence of FAD upon protein unfolding [16]. To demonstrate the capability of this assay in identifying compounds that bind to AfUGM, we measured the effect of substrates, products, and analogs on the T_m value. In the absence of ligands, the T_m value was 56.9 ± 0.05 °C. In the presence of 10 mM UDP, UDP-Gal*p*, or $NADP^+$, the T_m value increased by 3 °C, 0.5 °C, and 0.6 °C, respectively. Binding of these ligands was also confirmed using the ITC method (Table 1 and Fig. S1). For the HTS, AfUGM incubated with 10 mM UDP (2% DMSO final concentration) was used as the positive control and AfUGM in the presence of 2% DMSO was the negative control. The average Z′ for the HTS was 0.59 ± 0.02. The Selleckchem Kinase Inhibitor Library, which contains 273 structurally diverse inhibitors, was screened using the ThermoFAD assay. Compounds that increased the T_m of UGM by at least 1.5 °C were considered hits (Fig. S2). Following this criterion, four compounds were identified as hits. Three of these compounds were identified as false positives after close analysis of their thermal denaturation curves, which showed that assay artifacts resulted in a two-transition state denaturation effect (Fig. S3). Thus, only one compound, flavopiridol, was considered for further studies (Fig. 2A). In the presence of 200 μM of flavopiridol, the T_m value for AfUGM increased by 1.5 °C (Fig. 2B), indicating the formation of a stable complex [18]. Furthermore, the stabilization effect of flavopiridol is proportional to the ligand concentration (Fig. 2C).

3.2. IC_{50} and K_D

We confirmed that flavopiridol inhibits the reaction catalyzed by AfUGM in a concentration-dependent manner (Fig. 2D). Following the effect of flavopiridol on the initial velocity of conversion of UDP-Gal*f* to UDP-Gal*p*, monitored using UPLC, allowed for the determination of an IC_{50} value of 125 ± 5 μM. The K_D values for flavopiridol, UDP, $NADP^+$, and UDP-Gal*p* were calculated using ITC. The values are comparable with the K_D values obtained from the plots of T_m as a function of ligand concentration (Fig. 1C and Fig. S1A-C). ITC measurements allowed determination that the binding of flavopiridol to the oxidized AfUGM was enthalpy driven (Table 1, Fig. 2E) with a calculated K_D value of 38 ± 1 μM. Binding of flavopiridol to the reduced UGM was also enthalpy driven and about four times lower than the K_D for the oxidized UGM (Table 1, Fig. 2F).

3.3. Mechanism of inhibition

The activity of AfUGM as a function of UDP-Gal*f* concentration was measured in the absence or presence of flavopiridol (250 and 500 μM) by following the formation of UDP-Gal*p* from UDP-Gal*f* catalyzed by UGM under reducing conditions. No inhibition of MtUGM was observed under the same assay conditions (Fig. S4). The double reciprocal of the data best fit a non-competitive inhibitor equation and resulted in a K_i value of 454 ± 84 μM (Fig. 3A). Docking studies with the reduced UGM suggested that flavopiriol binds in the adenine-galactopyranose pocket (Fig. 3B) and interacts with several residues (Fig. 3C).

4. Discussion

The activity of UGM is important for the biosynthesis of galactofuran-containing molecules in many microbes. UGM is essential for *Mycobacterium tuberculosis* [8] and a virulence factor in *A. fumigatus* [12]. Its role in virulence and the absence of a UGM homolog in mammals has spurred the search and development of inhibitors against this enzyme [21,22]. Several methods have been used to screen inhibitors of UGMs mainly from bacterial sources [23]. A low throughput radiation-based assay was initially reported where UDP-[6-^{3}H]Gal*p* was used as substrate. In this assay, the reaction was allowed to proceed until 5% conversion was achieved (equilibrium occurs at 7% conversion). After treatment with periodate, only the product, UDP-[6-^{3}H]Gal*f*, results in tritiated formaldehyde, which must be isolated and quantified by scintillation counting. This assay was very laborious and time consuming [24]. Later, a fluorescence anisotropy assay was developed based on a UDP analog containing a fluorescein chromophore [25]. This assay was optimized for HTS format and was used to identify potent inhibitors of bacterial UGM. Unfortunately, this assay could not be used with eukaryotic UGMs (eUGMs) because the UDP-fluorescein had very low affinity for these enzymes. Although, the bacterial and

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Table 1
Thermodynamic parameters for binding of different ligands to AfUGM. K_D is the dissociation constant calculated with the ITC assay. K_D (tFAD) corresponds to the K_D obtained with the melting temperatures at various flavopiriol concentrations with the ThermoFAD assay.

Ligand	ΔH (kcal·mol^{-1})	K_ax10^4 (M^{-1})	ΔG (kcal·mol^{-1})	TΔS (kcal·mol^{-1})	K_D (μM)	K_D (tFAD) (μM)
$NADP^+$	−13.0 ± 0.3	2.4 ± 0.1	−4.6 ± 0.3	−8.4 ± 0.7	413 ± 24	199 ± 66
UDP	3.3 ± 0.1	18.4 ± 2.6	−7.1 ± 0.1	10.5 ± 0.3	5.4 ± 0.8	3.0 ± 0.1
UDP-Gal*p*	−0.3 ± 1	0.11 ± 0.08	−4 ± 2	3 ± 20	925 ± 690	600 ± 300
Flavopiriol	−5.3 ± 0.1	2.6 ± 0.9	−6.0 ± 0.1	0.71 ± 0.02	38 ± 1	154 ± 35
Flavopiriol[a]	−4.5 ± 0.1	9 ± 1	−6.8 ± 0.1	2.2 ± 0.1	10 ± 1	–

[a] UGM and flavopiriol were reduced with 5 mM sodium dithionite under anaerobic conditions.

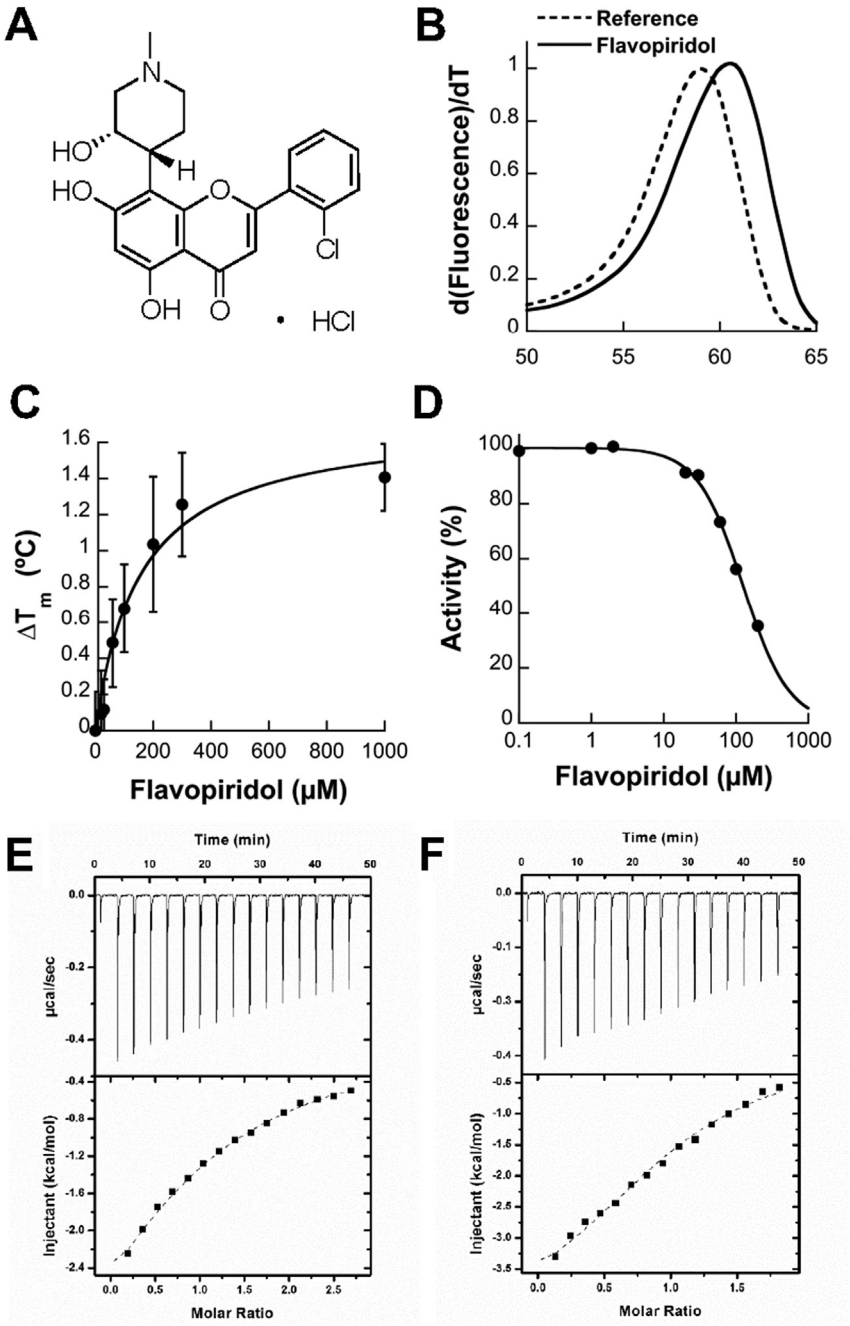

Fig. 2. Characterization of the inhibition mechanism of flavopiriodol and ITC results of binding. (A) Structure of flavopiridol. (B) Melting curve of UGM with (solid line) and without (dashed line) 200 μM flavopiridol. (B) Concentration dependence of the melting temperature of UGM with flavopiridol. (D) IC_{50} curve of inhibition of UGM by flavopiridol. ITC results of binding of flavopiridol to *A. fumigatus* UGM in the (E) oxidized and (F) reduced state. Top panels correspond to the heat released during the titration, while the bottom panels correspond to the integrated heat as a function of the reaction stoichiometry.

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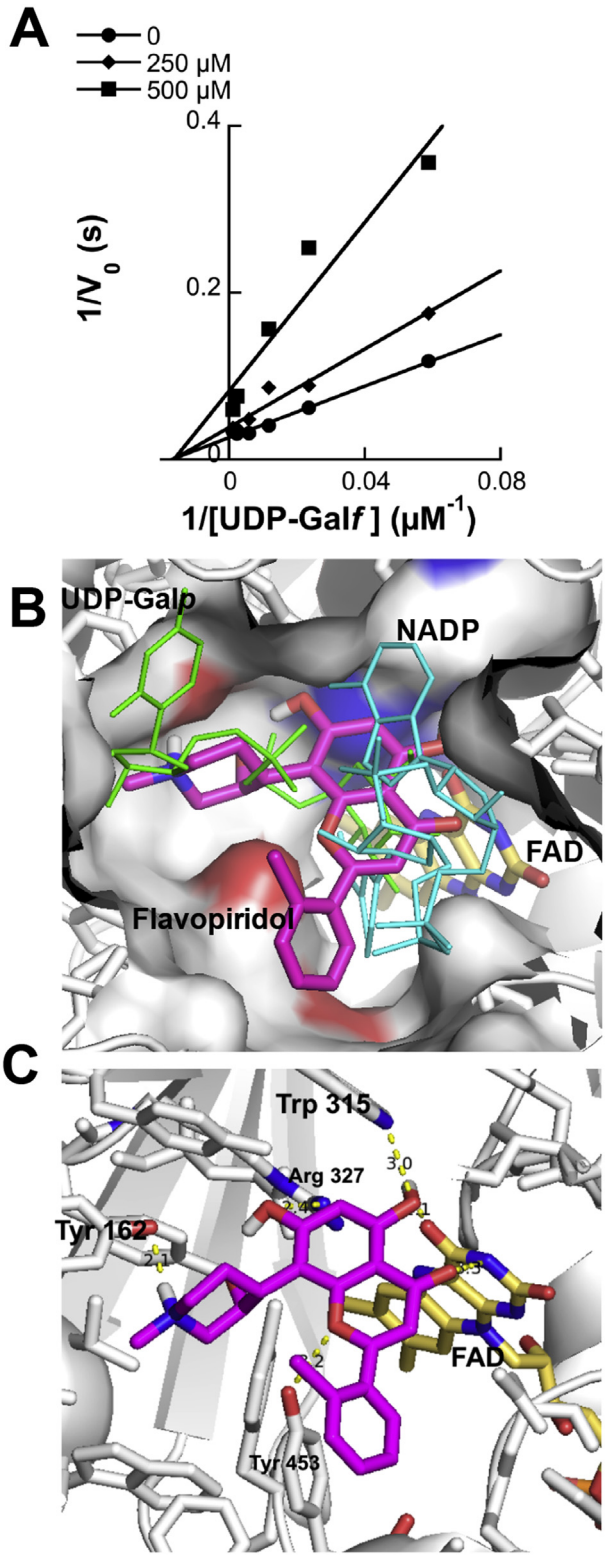

Fig. 3. Mechanism of inhibition of flavopiridol. (A) Double reciprocal curves for the inhibition of AfUGM by flavopiridol. Initial velocities were obtained by quantifying the production of UDP-Gal*p* from different concentrations of UDP-Gal*f* in the presence of AfUGM reduced with sodium dithionite. (B) Best docking pose of flavopiridol (magenta) in the NADP (cyan, PDB 5VWT) and UDP-Gal*p* (green, PDB 3UTH) binding site. (C) Predicted hydrogen bonding interactions (dotted yellow line) between the best docked pose of flavopiridol (magenta), FAD (yellow), and AfUGM (gray). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eukaryotic UGMs share a common fold, these enzymes are only 15% identical and differ significantly in molecular size, with eUGMs being 100 aminoo acids larger [26,27]. eUGMs have loops that undergo conformational changes during reduction and substrate binding [14,28,29]. These structural differences might be responsible for the low binding affinity of the UDP-fluorescein chromophore. We wanted to determine if the ThermoFAD assay could be used to identify compounds that bound to eUGMs. ThermoFAD is a differential scanning calorimetry assay that can be used to determine the T_m of flavo-proteins by monitoring the increase in FAD fluorescence upon unfolding [16]. Protein thermal stabilization assays are well-established methods to screen for enzyme binding ligands [18]. ThermoFAD has been successfully used to identify inhibitors of the FAD-dependent lipid-generating enzyme alkylglycerone phosphate synthase with K_D values of 200–700 nM [30]. We first determined if addition of substrates and other analogs provided a measurable shift in the T_m value that would indicate binding to the active site of AfUGM. We observed a concentration-dependent shift to higher T_m values in the presence of UDP, UDP-Galp, and $NADP^+$ (Fig. S1). Having established that the ThermoFAD assay could be used to identify compounds that bind to the active site of AfUGM, the assay was used to screen a small library of known kinase inhibitors. From this assay, flavopiridol was identified as a potential inhibitor of AfUGM as the T_m value increased 1.5 °C when 200 μM was used in the assay (Fig. S2). Flavopiridol is a semi-synthetic flavone derived from the natural alkaloid rohitukine [31], which has inhibitory activity against cyclin-dependent kinase [32]. Flavopiridol recently completed a Phase II clinical trial for treatment of acute myeloid leukemia [33]. Further characterization of flavopiridol showed that it inhibited the activity of AfUGM with an IC_{50} value of ~125 μM. This compound has no inhibitory activity with MtUGM, suggesting that flavopiridol might be specific to eUGMs. The K_D value (10 μM) for the active, reduced form of AfUGM is ~ 4-times lower than for the reduced form. The higher affinity of the flavopiridol for the reduced (active) form of AfUGM can be a consequence of the several conformational changes that occur upon reduction [14]. Docking studies show that flavopiridol forms hydrogen bonds with W315A and R327. These residues have been shown to undergo conformational changes upon FAD reduction. Flavopiriol was shown to be non-competitive against UDP-Galp and docking studies suggest that it binds in the shared NADP-UDP-Gal*p* site [34]. Together, the results presented here validate the ThermoFAD as a HTS assay for the identification of eukaryotic UGMs inhibitors.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.09.074.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.09.074.

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