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A fluorescence polarization binding assay to identify inhibitors of flavin-dependent monooxygenases

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ABSTRACT

N-Hydroxylating monooxygenases (NMOs) are essential for pathogenesis in fungi and bacteria. NMOs catalyze the hydroxylation of sine and ornithine in the biosynthesis of hydroxamate-containing siderophores. Inhibition of kynurenine monooxygenase (KMO), which catalyzes the conversion of kynurenine to 3-hydroxykynurenine, alleviates neurodegenerative disorders such as Huntington's and Alzheimer's diseases and brain infections caused by the parasite *Trypanosoma brucei*. These enzymes are examples of flavin-dependent monooxygenases, which are validated drug targets. Here, we describe the development and optimization of a fluorescence polarization assay to identify potential inhibitors of flavin-dependent monooxygenases. Fluorescently labeled ADP molecules were synthesized and tested. An ADP-TAMRA chromophore bound to KMO with a K_d value of 0.60 ± 0.05 μM and to the NMOs from *Aspergillus fumigatus* and *Mycobacterium smegmatis* with K_d values of 2.1 ± 0.2 and 4.0 ± 0.2 μM, respectively. The assay was tested in competitive binding experiments with substrates and products of KMO and an NMO. Furthermore, we show that this assay can be used to identify inhibitors of NMOs. A Z' factor of 0.77 was calculated, and we show that the assay exhibits good tolerance to temperature, incubation time, and dimethyl sulfoxide concentration.

Flavin-dependent monooxygenases constitute a large family of enzymes that catalyze a variety of chemical reactions, including epoxidation, hydroxylation, dehalogenation, aromatic hydrocarbon hydroxylation, and sulfur and nitrogen hydroxylation [1,2]. The activities of these enzymes are of significant importance to human health and chemical applications. In mammals, the structurally and biochemically well-characterized flavin monooxygenases (FMOs)[1] are involved in the biodegradation of xenobiotics in the liver [3,4]. The less characterized *N*-hydroxylating monooxygenases (NMOs) are involved in the biosynthesis of siderophores in fungi and bacteria. NMO catalyzes the NAD(P)H- and oxygen-dependent hydroxylation of the side chain of lysine and ornithine to form the modified N^6- and N^5-hydroxy amino acids, respectively (Scheme 1) [5,6]. Siderophores are essential for iron acquisition during infection by many human pathogens such as *Aspergillus fumigatus* and *Mycobacterium tuberculosis* [7,8]. The ornithine hydroxylase from *A. fumigatus*, known as *Af* SidA, is essential for virulence [9]. *M. tuberculosis* contains an N^6-lysine hydroxylase, known as MbtG, which has also been proposed as a potential drug target [7,10]. Recently, it was shown that inhibiting kynurenine monooxygenase (KMO) activity improves the pathology of Huntington's and Alzheimer's diseases and African trypanosomiasis [11–13]. KMO is also a flavin-dependent monooxygenase that catalyzes the conversion of kynurenine to 3-hydroxykynurenine (Scheme 1) [13,14].

The important roles of these flavin-dependent monooxygenases in microbial pathogen growth, neurodegenerative diseases, and parasitic infections show that they are important drug targets. Here, we present the development and application of a fluorescence polarization binding assay to identify small molecule inhibitors of flavin-dependent monooxygenases. Because NADPH is a common substrate in all of these enzymes, we designed an ADP-based fluorescently labeled ligand that has affinity to several monooxygenases. It was shown that substrates and products displace the ADP chromophore, indicating that the chromophore binds at the active site of both *Af* SidA and KMO. A screen of a small molecular library was performed, and an inhibitor of *Af* SidA was identified. Furthermore, we show that this assay has a Z' factor of 0.77 ± 0.01 and displays good temperature and dimethyl sulfoxide (DMSO) tolerance. More important, we show that this assay can be generally applied to other flavin-dependent monooxygenases.

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[1] *Abbreviations used:* FMO, flavin monooxygenase; NMO, *N*-hydroxylating monooxygenase; KMO, kynurenine monooxygenase; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; HRMS, high-resolution mass spectra; RT, room temperature; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; DMF, dimethylformamide.

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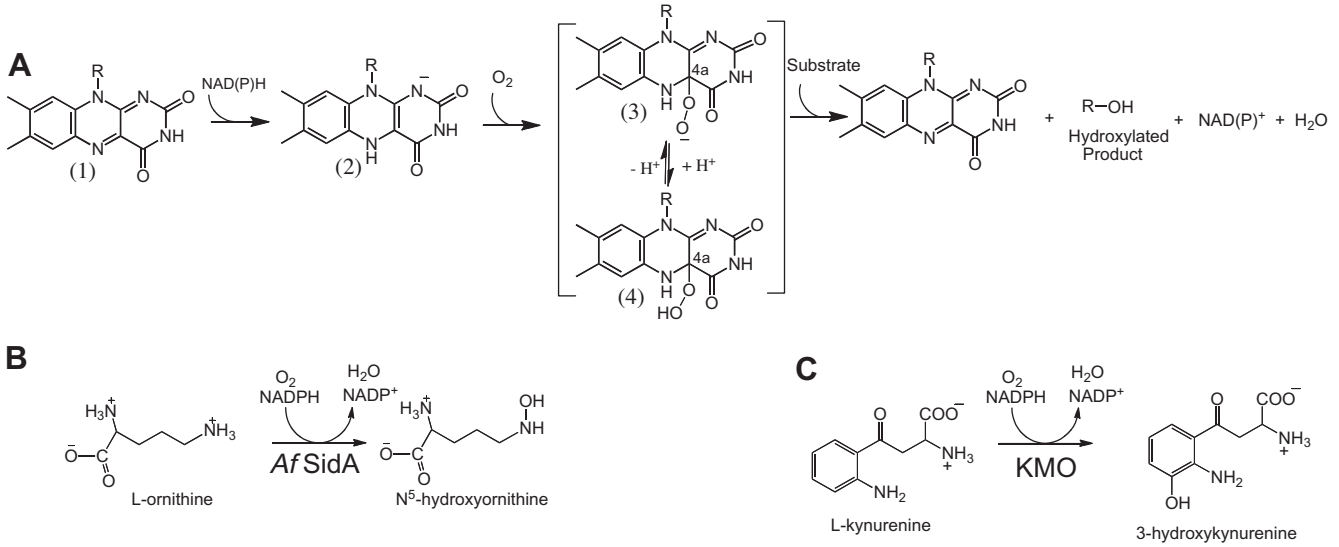


Scheme 1. Flavin-dependent monooxygenases. (A) Reaction of oxidized flavin (**1**) with NAD(P)H to form the reduced flavin (**2**). Upon reaction with molecular oxygen, the C4a–peroxyflavin (**3**) is formed and is stabilized by Baeyer–Villiger monooxygenases. Protonation of the C4a–peroxyflavin results in the formation of the C4a–hydroperoxyflavin (**4**), which is stabilized by hydroxylases. (B) Reaction catalyzed by *Af* SidA. (C) Reaction catalyzed by KMO.

Materials and methods

Materials

All reagents were obtained from commercial sources and were used without further purification. All solvents were either reagent grade or high-performance liquid chromatography (HPLC) grade. Anhydrous reactions were performed under argon. Nuclear magnetic resonance (NMR) spectral data were obtained using a Varian Inova spectrometer at 400 MHz. Chemical shifts were reported as δ values relative to known solvent residue peaks. High-resolution mass spectra (HRMS) were obtained from the Mass Spectrometry Incubator in the Department of Biochemistry at Virginia Tech. HPLC (Shimadzu LC 20A Prominence) was performed on a C18 reverse-phase column (Phenomenex Luna C18 column, 250 × 21.20 mm, 5 μm, and Phenomenex Luna C18 (2) column, 250 × 4.6 mm, 5 μm) using water and acetonitrile as the elution solvents. All compounds were more than 95% pure as judged by HPLC and ^{1}H NMR.

Protein expression and purification

Expression and purification of *Af* SidA was performed as described previously [5]. Expression and purification of *Mycobacterium smegmatis* N^6-lysine hydroxylase (MbsG, ~75% identical to the *M. tuberculosis* enzyme, MbtG) was performed as described previously [15]. The synthetic FMO gene from *Methylophaga* sp. strain SK1 was obtained from GenScript (Piscataway, NJ, USA) and cloned into the pET55A vector. The protein was expressed and purified following the procedures of Oppenheimer and coworkers [16]. Kynurenine 3-monooxygenase from *Pseudomonas fluorescens* was a generous gift from Dr. Graham Moran (University of Wisconsin–Milwaukee) [13].

Synthesis of ADP chromophores

*AMP triethylammonium salt **1***

Dowex 50WX8-200 (H^+) resin (5 g) in Et_3N (7 ml) and H_2O (43 ml) was stirred at room temperature (RT) for 5 h. After filtration, the resin was washed with H_2O and dried *in vacuo* to give Dowex 50WX8-200 (Et_3NH^+) resin. This resin (2 g) was added to a solution of AMP (673 mg, 1.72 mmol) in H_2O (10 ml), and the suspension was stirred at RT overnight before filtration and concentration to give triethylammonium salt **1** (800 mg, 99%). ^{1}H NMR (400 MHz, D_2O) δ 8.53 (s, 1H), 8.23 (s, 1H), 6.11 (d, J = 6.1 Hz, 1H), 4.77–4.74 (m, 1H), 4.48 (dd, J = 5.1, 3.4 Hz, 1H), 4.38–4.34 (m, 1H), 4.04 (dd, J = 4.7, 3.0 Hz, 2H), 3.18 (q, J = 7.3 Hz, 6H), 1.25 (t, J = 7.3 Hz, 9H) (see Fig. S1 in Supplementary material).

*ADP-linker conjugate **3***

Dimethylpyridine (114 μl, 0.9 mmol), Et_3N (63 μl, 0.45 mmol), and trifluoroacetic anhydride (1 ml, 1.4 M in acetonitrile) were added dropwise at 0 °C to a suspension of AMP triethylammonium salt **1** (100 mg, 0.22 mmol) in acetonitrile (3 ml). The resulting red–brown solution was stirred for 15 min before being concentrated *in vacuo* and redissolved in acetonitrile (3 ml). After successive addition of molecular sieves (4 Å, 100 mg), Et_3N (153 μl, 1.1 mmol), and methylimidazole (968 μl, 1.2 mmol) at 0 °C, a solution of phosphate **2** (70 mg, 0.18 mmol) in acetonitrile (1 ml) was added dropwise to the suspension at 0 °C, and the suspension was stirred at 0 °C for 1 h and at RT for 3 h. The suspension was then filtered and washed with H_2O. The filtrate was concentrated and purified by silica gel flash chromatography ($CHCl_3$/MeOH/1 M NH_4OAc = 5:4:1) to give ADP conjugate **3** (70 mg, 0.11 mmol, 50%). ^{1}H NMR (400 MHz, D_2O) δ 8.49 (s, 1H), 8.18 (s, 1H), 6.09 (d, J = 5.6 Hz, 1H), 4.73 (t, J = 5.4 Hz, 1H), 4.50 (t, J = 4.2 Hz, 1H), 4.36 (s, 1H), 4.20 (s, 2H), 3.80 (d, J = 6.3 Hz, 2H), 3.11 (t, J = 7.1 Hz, 2H), 1.45–1.37 (m, 2H), 1.36–1.27 (m, 2H), 1.14–1.02 (m, 4H). HRMS (matrix-assisted laser desorption/ionization time-of-flight [MALDI–TOF]): calcd. for $C_{18}H_{26}F_3N_6O_{11}P_2$ $(M–H)^-$ 621.1087, found 621.1071 (see Fig. S2).

*ADP-conjugated amine **4***

ADP-linker conjugate **3** (35 mg, 0.056 mmol) was dissolved in 3 M NH_4OH (5 ml), and the resulting solution was stirred at RT for 2 h. After being concentrated *in vacuo*, the residue was redissolved in H_2O and lyophilized to give amine **4** as a white powder (30 mg, 0.055 mmol, 98%). ^{1}H NMR (400 MHz, D_2O) δ 8.50 (s, 1H), 8.23 (s, 1H), 6.11 (d, J = 6.2 Hz, 1H), 4.72–4.69 (m, 1H), 4.54–4.49 (m, 1H), 4.39–4.34 (m, 1H), 4.21–4.17 (m, 2H), 3.84–3.78 (m, 2H), 2.89 (t, J = 7.5 Hz, 2H), 1.58–1.37 (m, 4H), 1.22–1.66 (m, 4H). HRMS (MALDI–TOF): calcd. for $C_{16}H_{27}N_6O_{10}P_2$ $(M–H)^-$ 525.1264, found 525.1225 (see Fig. S3).

*Chromophore **1***. A solution of rhodamine SE (1 mg, 2.0 μmol) in dimethylformamide (DMF, 50 μl) was added to a solution of amine

4 (1.5 mg, 2.7 μmol) in $NaHCO_3$ buffer (50 μl, 0.1 M, pH 8.3) at RT. After stirring at RT for 2 h, the solution was diluted with H_2O (1.9 ml) and acetonitrile (0.1 ml), injected onto a reverse-phase HPLC column (Phenomenex Luna C18 (2) column, 250 × 4.6 mm, 5 μm), and purified at a flow rate of 1.0 ml/min with linear gradient elution (5–65% acetonitrile in H_2O over 55 min, then 65–95% over 5 min) to afford chromophore **1** (1.2 mg, 69%). HRMS (MALDI–TOF): calcd. for $C_{37}H_{39}N_8O_{14}P_2$ $(M–H)^-$ 881.2061, found 881.1163 (see Fig. S4).

*Chromophore **2**.* A solution of 5(6)-rhodamine-X SE (1 mg, 1.6 μmol) in DMF (50 μl) was added to a solution of amine **4** (1.0 mg, 1.8 μmol) in $NaHCO_3$ buffer (50 μl, 0.1 M, pH 8.3) at RT. After stirring at RT for 2 h, the solution was diluted with H_2O (1.9 ml) and acetonitrile (0.1 ml), injected onto a reverse-phase HPLC column (Phenomenex Luna C18 (2) column, 250 × 4.6 mm, 5 μm), and purified at a flow rate of 1.0 ml/min with linear gradient elution (5–65% acetonitrile in H_2O over 55 min, then 65–95% over 5 min) to afford chromophore **2** (1.0 mg, 62%). HRMS (MALDI–TOF): calcd. for $C_{43}H_{50}N_9O_{15}P_2$ $(M–H)^-$ 994.2902, found 994.1824 (see Fig. S5).

*Chromophore **3** and chromophore **4***

A solution of 5(6)-TAMRA-X SE (1 mg, 1.6 μmol) in DMF (50 μl) was added to a solution of amine **4** (1.0 mg, 1.8 μmol) in $NaHCO_3$ buffer (50 μl, 0.1 M, pH 8.3) at RT. After stirring at RT for 5 h, the solution was diluted with H_2O (1.9 ml) and acetonitrile (0.1 ml), injected onto a reverse-phase HPLC column (Phenomenex Luna C18 column, 250 × 21.50 mm, 5 μm), and purified at a flow rate of 5.0 ml/min with linear gradient elution (5–30% acetonitrile in H_2O over 30 min, then 30–95% over 10 min) to afford 6-TAMRA chromophore **3** (0.5 mg, 30%) and 5-TAMRA chromophore **4** (0.6 mg, 37%).

*Chromophore **3**.* ^1H NMR (400 MHz, D_2O) δ 8.38 (s, 1H), 8.02 (dd, J = 8.1, 1.7 Hz, 1H), 7.93 (d, J = 8.1 Hz, 1H), 7.86 (s, 1H), 7.82 (d, J = 1.8 Hz, 1H), 7.15 (d, J = 9.6 Hz, 1H), 7.10 (d, J = 9.6 Hz, 1H), 6.89 (dd, J = 9.5, 2.3 Hz, 1H), 6.85–6.80 (m, 1H), 6.69–6.64 (m, 2H), 5.77 (d, J = 4.6 Hz, 1H), 4.45 (dt, J = 15.7, 4.9 Hz, 2H), 4.25–4.20 (m, 1H), 4.19–4.14 (m, 2H), 3.76–3.67 (m, 2H), 3.46 (t, J = 6.2 Hz, 2H), 3.25 (s, 6H), 3.23 (s, 6H), 2.81 (td, J = 6.3, 2.6 Hz, 2H), 2.15 (t, J = 7.0 Hz, 2H), 1.70–1.61 (m, 2H), 1.61–1.52 (m, 2H), 1.40–1.30 (m, 2H), 1.30–1.20 (m, 2H), 1.07–0.94 (m, 2H), 0.90–0.78 (m, 4H). HRMS (MALDI–TOF): calcd. for $C_{47}H_{58}N_9O_{15}P_2$ $(M–H)^-$ 1050.3528, found 1050.3514 (see Fig. S6).

*Chromophore **4**.* ^1H NMR (400 MHz, D_2O) δ 8.23–8.20 (m, 2H), 8.10 (dd, J = 8.1, 1.8 Hz, 1H), 8.02 (s, 1H), 7.45 (d, J = 7.8 Hz, 1H), 7.14 (d, J = 9.4 Hz, 1H), 7.01 (d, J = 9.4 Hz, 1H), 6.91 (dd, J = 9.7, 2.5 Hz, 1H), 6.81–6.74 (m, 2H), 6.61 (d, J = 2.5 Hz, 1H), 5.74 (d, J = 4.9 Hz, 1H), 4.38–4.35 (m, 2H), 4.15–3.95 (m, 3H), 3.86–3.82 (m, 2H), 3.50 (t, J = 6.6 Hz, 2H), 3.26 (s, 6H), 3.20 (s, 6H), 3.07 (t, J = 7.0 Hz, 2H), 2.27 (t, J = 6.8 Hz, 2H), 1.74–1.64 (m, 4H), 1.49–1.37 (m, 4H), 1.36–1.27 (m, 2H), 1.16–1.07 (m, 4H). HRMS (MALDI–TOF): calcd. for $C_{47}H_{58}N_9O_{15}P_2$ $(M–H)^-$ 1050.3528, found 1050.3481 (see Fig. S7).

Fluorescence polarization binding assay

Solutions containing serially diluted *Af* SidA, KMO, FMO, or MbsG and 30 nM chromophore **4** in 0.05 M sodium phosphate buffer (pH 7.0) were incubated at RT for 5 min. Each experiment was done in triplicate in a 96-well black half-area flat-bottom plate (Corning, Corning, NY, USA) at a final volume of 25 μl. The anisotropy values were measured (excitation at 544 nm and emission at 584 nm) on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The K_d values were obtained by fitting the anisotropy data to Eq. (1), where m_1 and m_2 are the minimum and maximum anisotropy values, respectively, m_3 is the K_d value, and C_t represents the total concentration of ADP chromophore:

$$y = m_1 + (m_2 - m_1)\frac{(x + C_t + m_3) - \sqrt{(x + C_t + m_3)^2 - 4xC_t}}{2C_t} \quad (1)$$

Fluorescence polarization displacement assay

Solutions (25 μl) containing 2 μM *Af* SidA or 0.6 μM KMO and 30 nM chromophore **4** in 0.05 M sodium phosphate buffer (pH 7.0) were mixed with various concentrations of tested ligands (NADPH, $NADP^+$, NAD^+, ADP, lysine, ornithine, or kynurenine) and incubated at RT for 5 min. When NADPH was the ligand, the sample was tested immediately after mixing to minimize oxidation. Each assay was performed in triplicate. Anisotropy values were measured, and the K_d values were obtained by fitting the data to Eq. (2), where m_1 and m_2 are the minimum and maximum anisotropy values, respectively, m_3 is the slope, and m_4 is the K_d:

$$y = m_1 + \frac{(m_2 - m_1)x^{m_3}}{m_4^{m_3} + x^{m_3}} \quad (2)$$

Fluorescence polarization displacement assay in high-throughput screening

We screened 160 small molecule compounds from the Spectrum Collection (MicroSource Discovery Systems, Gaylordsville, CT, USA) using the fluorescence polarization displacement assay with *Af* SidA. Each sample contained 25 μl of 2 μM *Af* SidA, 30 nM chromophore **4**, and 10 μM test compound in 0.05 M sodium phosphate buffer (pH 7.0) at a DMSO concentration of 2% (v/v). The mixtures were incubated for 5 min and read on a plate reader. The anisotropy values were normalized to a negative control (2 μM *Af* SidA and 30 nM chromophore **4** in 0.05 M sodium phosphate, pH 7.5) at 100% signal. A background control containing 30 nM and chromophore **4** without enzyme was also included. A positive control containing 2 μM *Af* SidA, 30 nM chromophore **4**, and 50 μM $NADP^+$ was present in every plate. One positive hit (sanguinarine sulfate) was identified, its affinity for *Af* SidA was determined, and the inhibitory effect was evaluated in the ornithine hydroxylation assay. A negative hit, berberine (which has similar chemical structure to sanguinarine), was selected to see whether the binding was specific. The addition of up to 1 mM of this compound did not produce a decrease in anisotropy similar to sanguinarine (see Fig. S9). Similarly, the negative hit, naproxen, did not bind to *Af* SidA (see Fig. S9).

Activity assay and inhibition of Af SidA

The enzymatic activity of *Af* SidA was monitored by measuring the amount of N^5-hydroxyornithine formed as described previously [15]. To determine the IC_{50} value for sanguinarine sulfate, the activity of *Af* SidA (2 μM) in 100 mM potassium phosphate (pH 7.5) in the presence of 200 μM NADPH and 1 mM ornithine was determined at various concentrations of the inhibitor (100, 300, 500, 750, and 1000 μM) (see Fig. 5B).

Z′ factor versus time

A 25-μl solution containing 1 μM *Af* SidA and 30 nM chromophore **4** in 0.05 M sodium phosphate buffer (pH 7.0) was incubated in the presence of 30 μM ADP (positive control) or in the absence of ADP (negative control) and dispensed into 20 wells on a 96-well plate. The anisotropy value of each well was measured at the following time points: 10, 30, 60, 90, 120, 180, 240, 1080, and

1440 min. This experiment was repeated three times on 3 separate days. The Z' factor was calculated using Eq. (3), where μ_+ and σ_+ represent the mean and standard error of the positive control, respectively, and μ_- and σ_- represent the mean and standard error of the negative control, respectively:

$$Z' = 1 - \frac{3(\sigma_- + \sigma_+)}{\mu_- - \mu_+} \quad (3)$$

Z′ factor versus temperature

A solution containing 1 μM *Af* SidA and 30 nM chromophore **4** in 0.05 M sodium phosphate buffer (pH 7.0) in the presence of 30 μM ADP (positive control) or in the absence of ADP (negative control) was dispensed into 20 wells on a 96-well black plate with a final volume of 25 μl. The plate was incubated at various temperatures (15, 20, 25, 30, and 35 °C) for 10 min. This experiment was repeated three times on 3 separate days. The Z' factor was calculated using Eq. (3).

DMSO tolerance

A solution containing 1 μM *Af* SidA, 30 nM chromophore **4**, and DMSO at various concentrations (1%, 2%, 3%, 4%, 5%, 7%, 9%, 11%, 15%, and 20%, v/v) in 0.05 M sodium phosphate buffer (pH 7.0) in the presence of 30 μM ADP (positive control) or in the absence of ADP (negative control) was dispensed into 20 wells on a 96-well black plate with a final volume of 25 μl. The plate was incubated at RT for 5 min. This experiment was repeated three times on 3 separate days. The Z' factor was calculated using Eq. (3).

Results

Chromophore design and synthesis

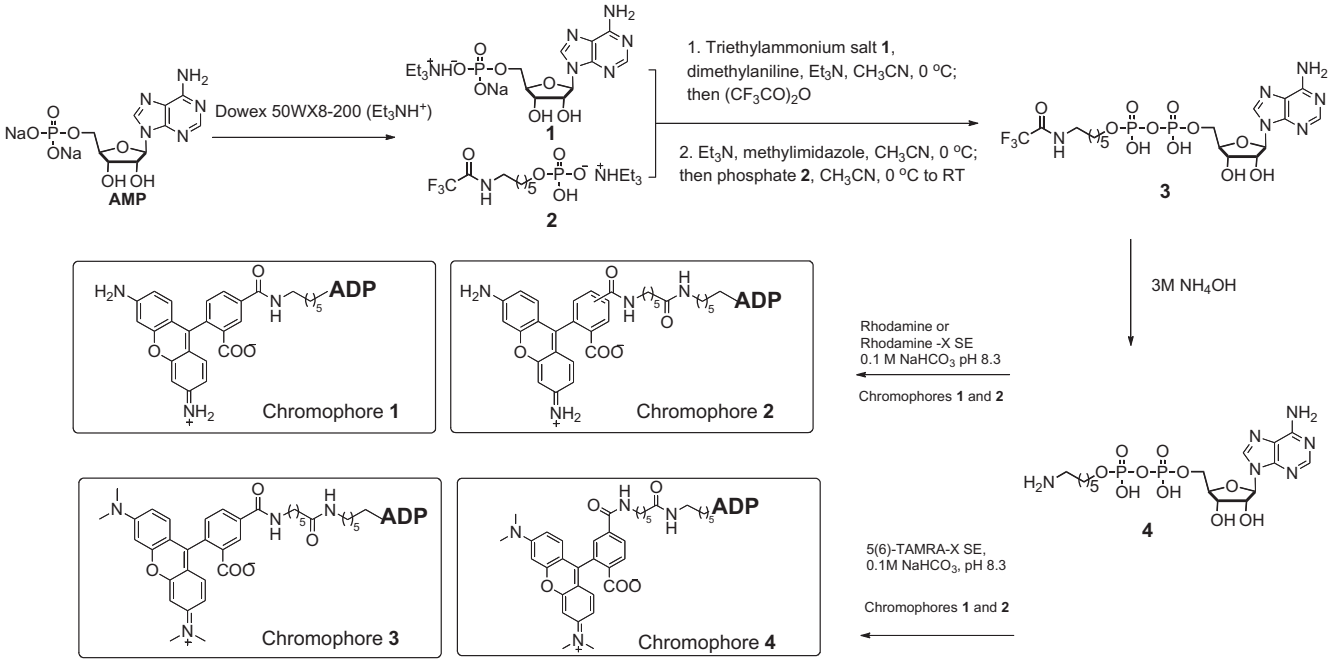
We used ADP as the backbone to synthesize a chromophore to target the NADPH binding site of flavin-dependent monooxygenases. A linker containing a free amine was added such that the resulting compound **4** could be easily reacted with various amine reactive fluorophores (Scheme 2). Four chromophores—ADP-rhodamine (chromophore **1**), ADP-rhodamine with an additional 5-carbon linker (chromophore **2**), and ADP-TAMRA linked to the 6-position (chromophore **3**) and 5-position (chromophore **4**)—were synthesized and isolated in 30% to 60% yield. The identity and purity of the compounds were determined by NMR and HRMS.

Table 1
Affinity of ADP chromophores to various flavin-dependent monooxygenases.

Chromophore	K_d (μM)			
	Af SidA	MbsG	FMO	KMO
1	>10	1.8 ± 0.2	>10	>10
2	0.4 ± 0.1	2.0 ± 0.2	>10	>10
3	9.0 ± 5.0	5.7 ± 0.7	13.0 ± 2.0	10.0 ± 2.0
4	2.1 ± 0.2	4.0 ± 0.2	6.0 ± 1.0	0.60 ± 0.05

Chromophore binding to flavin-dependent monooxygenases

Binding of each ADP chromophore was tested with purified recombinant FMOs (Table 1). Chromophore **1** had weak affinity for *Af* SidA, FMO, and KMO. In contrast, chromophore **2** had low micromolar affinity for *Af* SidA, whereas the affinity for FMO and KMO was unaffected. To find a "universal" flavin-dependent monooxygenases fluorescent ligand, we synthesized and tested ADP-TAMRA ligands (chromophores **3** and **4**). TAMRA is a derivative of rhodamine that contains a positively charged dimethylamino group. It is photostable and can be excited at 544 nm with the emission measured at 584 nm. Longer excitation and emission wavelengths decrease the interference signals from the flavin cofactor and other molecules [17]. Binding of chromophore **3** was detected for all flavin-dependent monooxygenases tested in this work. Chromophore **4** binds with higher affinity for all tested enzymes (Table 1 and Fig. 1); thus, we chose this fluorescent ligand for further characterization. Interestingly, the binding affinity for MbsG remained relatively constant independent of what ligand was used (Table 1).



Scheme 2. Synthesis of fluorescently labeled ADP chromophores.

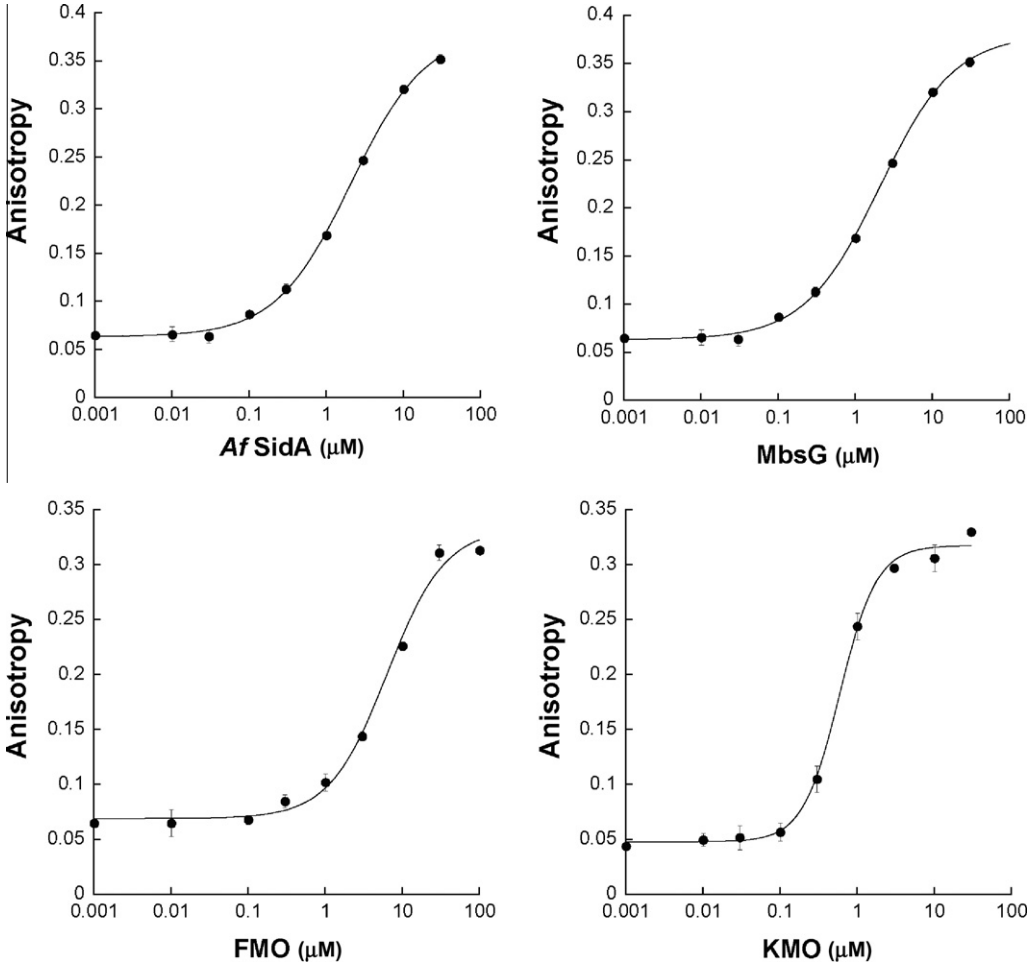


Fig.1. Binding curves of chromophore **4** (30 nM in 0.05 M sodium phosphate, pH 7.0) to various flavin-dependent monooxygenases.

Competitive displacement experiments

We further evaluated the binding of chromophore **4** to *Af* SidA and KMO. To determine whether chromophore **4** indeed was binding at the active site of these enzymes, competition experiments with substrates and products were performed. Fig. 2 shows a representative sample of the competition curves for $NADP^+$, NAD^+, ornithine, and lysine for *Af* SidA. The K_d values calculated from these experiments are summarized in Table 2. Competition by ADP was also tested, and the binding affinity was calculated (Table 2).

Determination of Z′ factor and fluorescence polarization assay optimization

The *Z′* factor is a statistical parameter that estimates the reliability and robustness of the assay [18]. A value between 0.5 and 1 corresponds to a good assay response. We calculated the *Z′* factor

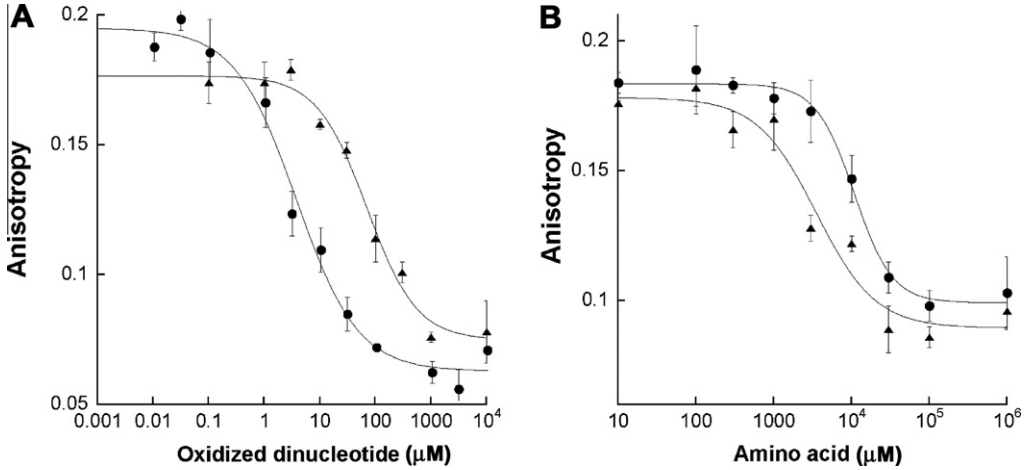


Fig.2. (A) Competitive binding of NAD^+ (triangles) and $NADP^+$ (circles) to *Af* SidA. (B) Competitive binding of ornithine (triangles) and lysine (circles) to *Af* SidA.

with chromophore **4** to be 0.77 ± 0.01 at 25 °C. The assay is resistant to temperature changes between 15 and 35 °C and to DMSO concentrations between 0% and 5% (Fig. 3). In addition, we tested the stability and accuracy of the assay in the presence of 2% DMSO, which is the concentration used in the initial screening conditions. The results show that the fluorescence anisotropy values in all wells vary less than 5% and are stable for more than 4 h after plating (see Fig. S8 in Supplementary material).

Identification of inhibitors of Af SidA

We tested whether binding of small molecules to *Af* SidA could be identified using the assay developed here. We screened 160 random compounds from the Spectrum Collection (MicroSource Discovery Systems). This library is composed of various known drugs, experimental bioactives, and pure natural products. Using 2 μM *Af* SidA and a fixed concentration of the compounds (10 μM), it was clear that several compounds did not decrease the anisotropy values, indicating that they did not compete for the binding site of the chromophore. One compound (sanguinarine sulfate) produced a decrease in the anisotropy value, but not as low as the positive control (containing 50 μM $NADP^+$) (Fig. 4). We determined a K_d value of 110 ± 13 μM for sanguinarine sulfate (MicroSource Discovery Systems, product No. 00310035). To determine whether this compound was able to inhibit the activity of *Af* SidA, the IC_{50} value was determined by measuring the amount of hydroxylated ornithine produced in the ornithine hydroxylation assay. Sanguinarine sulfate was found to be a weak inhibitor of *Af* SidA with an IC_{50} value of 500 ± 90 μM (Fig. 5).

Discussion

Flavin-dependent monooxygenases catalyze a number of chemical reactions. This chemical versatility originates from different active sites that accommodate a variety of substrates and from the modulation of the chemical nature of the oxygenated flavin intermediate [1,19]. In all flavin-dependent monooxygenases, the flavin cofactor must be reduced to react with molecular oxygen and form the C4a–flavin adduct (Scheme 1). If the protein catalyzes the nucleophilic addition of molecular oxygen, such as in the case of Baeyer–Villiger monooxygenases, the C4a–peroxyflavin is stabilized. If hydroxylation is the desired chemical outcome, the C4a–hydroperoxyflavin is stabilized. The hydroxylation products of several monooxygenases have been shown to be important for virulence in many human pathogens. For example, to proliferate and establish infection under iron-limiting conditions, such as those found in mammals, microbial pathogens must obtain iron from the host [1]. One mechanism of iron acquisition is via the use of iron chelators known as siderophores [20]. It has been shown that hydroxamate-containing siderophores in *M. tuberculosis* are essential for virulence. In *A. fumigatus*, the deletion of the sida gene results in a mutant strain that does not synthesize the hydroxamate-containing siderophore ferricrocin and is unable to establish infection in mice, which links the activity of a single protein to the virulence of this pathogenic fungus [9]. FMOs have also been identified as potential drug targets against parasitic and neurodegenerative diseases. It has recently been shown that targeted inhibition of KMO results in an increase in kynurenic acid, a neuroprotective compound in the brain. High levels of kynurenic acid alleviate neurodegeneration in mouse models of Alzheimer's and

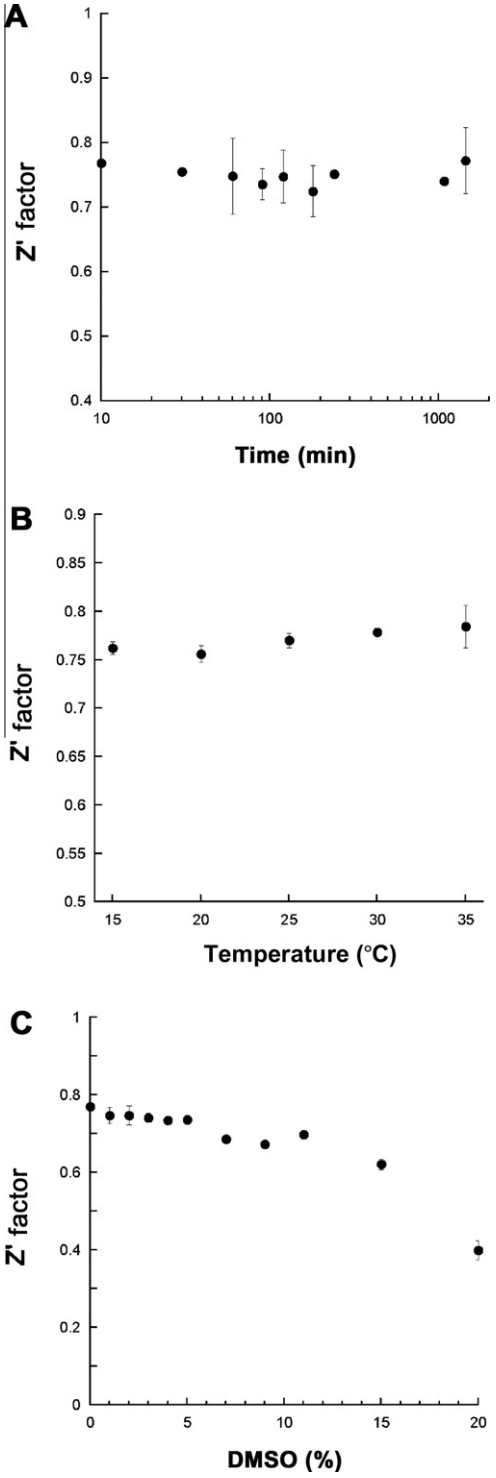


Fig.3. Changes in the *Z′* factor value as a function of time (A), temperature (B), and DMSO concentration (C).

Table 2
K_d values of substrates or inhibitor of *Af* SidA.

Ligand	*Af* SidA	KMO
NADPH	3.3 ± 0.1 μM	152 ± 80 μM
$NADP^+$	4 ± 1 μM	>10 mM
NAD^+	70 ± 20 μM	670 ± 70 μM
ADP	11 ± 1 μM	50 ± 10 μM
Lysine	11 ± 1 mM	n.d.
Ornithine	4 ± 1 mM	n.d.
Kynurenine	n.d.	180 ± 40 μM
Sanguinarine	110 ± 10 μM	n.d.

Note. n.d., not determined.

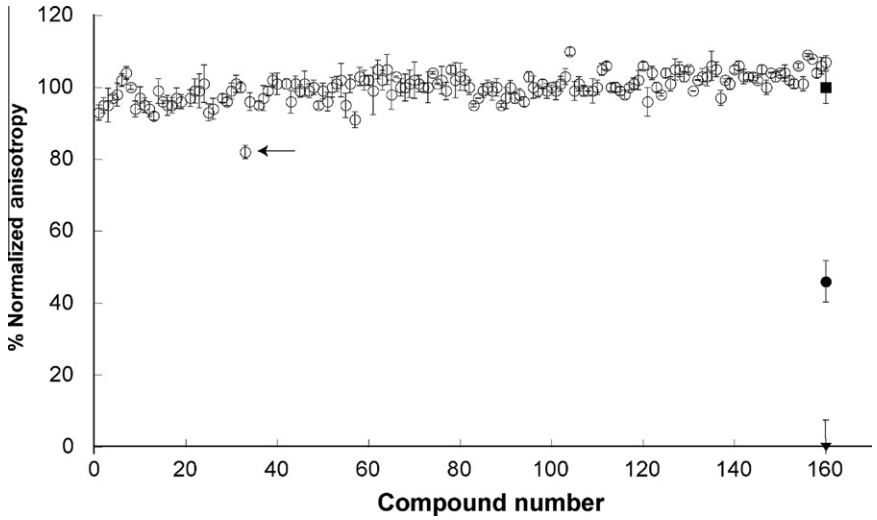


Fig.4. Identification of an *Af* SidA inhibitor. Binding of potential inhibitors to the active site of *Af* SidA was monitored by measuring the fluorescence anisotropy decrease as a consequence of the displacement of chromophore **4**. Open circles show the anisotropy values of the compounds tested. In the assay, we also included a negative control that contains only *Af* SidA–chromophore complex (solid square), a sample with 50 μM $NADP^+$ as the positive control (solid circle), and chromophore alone as the background (inverse solid triangle). The arrow points at the anisotropy value in well 32, which contains the compound sanguinarine. This molecule appears to bind *Af* SidA, as is evident by a slight decrease in the anisotropy value. Assay conditions are described in Materials and methods.

Huntington's diseases [11]. Similarly, targeted inhibition of KMO has been shown to diminish the pathology of African trypanosomiasis or sleeping sickness [12]. Thus, identification of inhibitors of flavin-dependent monooxygenases can result in lead compounds that may be developed into potential drugs for treating tuberculosis, aspergillosis, sleeping sickness, and Alzheimer's and Huntington's diseases.

The need for inhibitors of these enzymes and the potential health impact prompted us to develop an assay that can be used to perform high-throughput screening of chemical libraries. This assay must identify small molecule compounds that bind to the active site of these proteins, be sensitive, and use small amounts of protein and other materials to reduce cost. Because the only common substrate in all of these enzymes is NAD(P)H, we used this molecule as the building block for the design of a fluorescent ligand. In the reaction of all the enzymes tested here, the nicotinamide ring of NAD(P)H must bind close to the flavin in order to transfer a hydride from the C4-position of NAD(P)H to the flavin N5-position [21]. We reasoned that it could be possible to use the ADP portion of this substrate to function as a carrier molecule that will recognize the NAD(P)H binding site. To increase the affinity of the ligand, we selected a three-aromatic-ring chromophore that might interact with the flavin via aromatic stacking interactions. A linker was added to ADP to enhance flexibility and to allow the chromophore to reach and interact with the flavin. In our initial experiments, we used the chromophore rhodamine, which resulted in binding to only MbsG (Table 1). The addition of an extra 5-carbon linker (compound **2**) resulted in similar binding to MbsG and a significant increase in binding to *Af* SidA; however, very low affinity for FMO and KMO was observed. Therefore, we added the chromophore TAMRA, which we showed has high affinity to other flavin proteins [17]. In addition, low background fluorescence from the bound flavin and other aromatic compounds is obtained with TAMRA because it absorbs at 545 nm and emits at 584 nm [17]. Commercially available TAMRA is sold as a mixture of the aminohexanoyl spacer bound at the 5- and 6-positions of benzoic acid. We isolated each isomer and determined that the ADP-5-TAMRA (chromophore **4**) bound with high affinity to all of the enzymes (Table 1). Further characterization of the binding of chromophore **4** was performed with *Af* SidA and KMO. Because ADP was used as the building block of the chromophore, the addition of $NADP^+$,

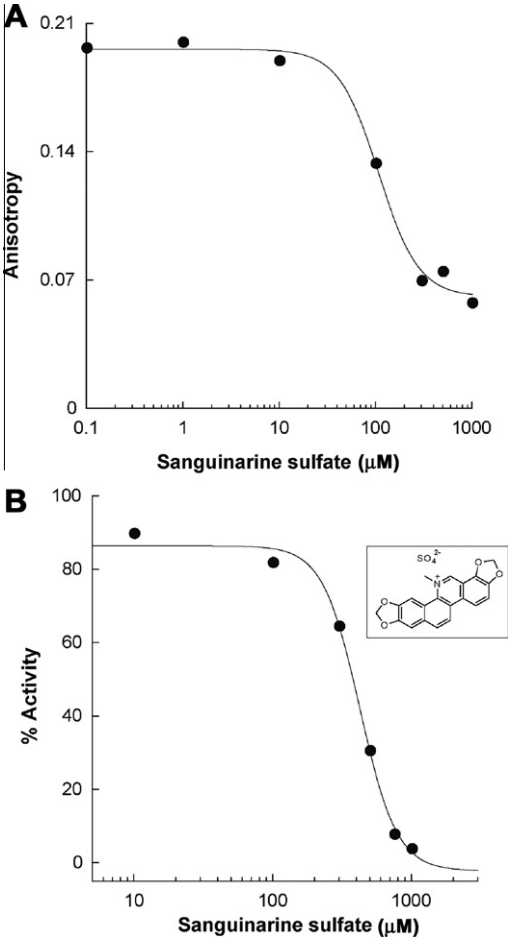


Fig.5. Determination of K_d and IC_{50} values of sanguinarine to *Af* SidA. (A) Anisotropy changes as a function of sanguinarine concentration were used to determine a K_d value of 110 ± 13 μM. (B) Inhibition of ornithine hydroxylation activity of *Af* SidA as a function of sanguinarine concentration. An IC_{50} value of 500 ± 90 μM was calculated.

NAD^+, and ADP should displace the chromophore from its binding site, resulting in a decrease in anisotropy. A decrease in anisotropy was observed when the concentrations of these ligands were increased, permitting the calculation of the K_d values (Table 2 and Fig. 2). The K_d values for NADPH, $NADP^+$, and NAD^+ of *Af* SidA are consistent with values previously determined using other methods [5,22]. KMO has very low affinity to $NADP^+$, and no reports of NAD^+ binding have been published; however, our K_d value for NADPH is consistent with the value reported in the literature (Table 2) [14]. These results suggest that chromophore **4** is binding in the NADPH binding site, as expected. We also tested whether chromophore **4** could be displaced from the active site of *Af* SidA and KMO by molecules that do not resemble NADPH. With *Af* SidA, we determined the binding of ornithine and the substrate analog, lysine [5]. Kynurenine was tested with KMO. Binding was determined by measuring the change in anisotropy as a function of increasing ligand concentrations (Table 2 and Fig. 2). Clearly, chromophore **4** can be displaced by both NADPH-like compounds and other small molecules that bind to the active site of these enzymes. The assay was further characterized, and it was shown to have a Z' factor value of 0.77 ± 0.01 and to display good temperature and DMSO tolerance, indicating that the assay is suitable for high-throughput screening. The ability of this assay to report on the binding of potential inhibitors of *Af* SidA was further tested by screening 160 low-molecular-weight compounds (Fig. 4) [17]. The screen clearly shows that 159 compounds did not bind to *Af* SidA because the anisotropy values were similar to the negative control. However, one compound decreased the anisotropy value by 20%, suggesting that this compound binds to the active site of *Af* SidA, albeit with low affinity. A K_d value of 110 ± 13 μM was calculated for this compound, which was identified as sanguinarine. We then determined whether this compound could inhibit the activity of *Af* SidA in the presence of NADPH and ornithine. An IC_{50} value of 500 ± 90 μM was calculated, and 100% inhibition was observed (Fig. 4). These data clearly show that small molecule compounds that inhibit *Af* SidA can be identified using this assay, even those with low affinity. Sanguinarine has been previously described as an inhibitor of cancer cell proliferation by a depolymerizing effect on cellular microtubules [23]. This compound is also reported to be a potent inhibitor of mitogen-activated protein kinase phosphatase-1 (MKP-1) [24]. Thus, it appears that it has some affinity to ATP binding enzymes. The identification of sanguinarine as a ligand of *Af* SidA is consistent with the fact that this enzyme also binds nucleotides. We also showed that this assay can be generally used for the identification of other flavin-dependent monooxygenases given that chromophore **4** can effectively bind to MbsG and KMO. Thus, inhibitors that can be used to treat diseases such as tuberculosis and Alzheimer's and Huntington's diseases can be obtained using the assay presented here.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.03.002.

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