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Development of a Pterin-based Fluorescent Probe for Screening Dihydropteroate Synthase

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Abstract

Dihydropteroate synthase (DHPS) is the classical target of the sulfonamide class of antimicrobial agents, whose use has been limited by widespread resistance and pharmacological side effects. We have initiated a structure-based drug design approach for the development of novel DHPS inhibitors that bind to the highly conserved and structured pterin sub-site rather than to the adjacent *p*-amino benzoic acid binding pocket that is targeted by the sulfonamide class of antibiotics. To facilitate these studies, a robust pterin site-specific fluorescence polarization (FP) assay has been developed and is discussed herein. These studies include the design, synthesis and characterization of two fluorescent probes, and the development and validation of a rapid DHPS FP assay. This assay has excellent DMSO tolerance and is highly reproducible as evidenced by a high *Z'* factor. This assay offers significant advantages over traditional radiometric or phosphate release assays against this target, and is suitable for site-specific high throughput and fragment-based screening studies.

Introduction

There is a clear need to develop novel antimicrobial agents due to the widespread emergence of drug resistance.^{1, 2} Sulfonamides have been used as synthetic antibiotics since the 1930s to treat a wide variety of Gram-positive, Gram-negative and protozoal infections, but their use has been greatly compromised by the emergence of resistance and the poor tolerance of certain patient populations.^{2–7} Sulfonamides target the enzyme dihydropteroate synthase (DHPS) encoded by the *folP* gene and function as competitive inhibitors of one of the DHPS substrates, *p*-amino benzoic acid (*p*ABA).⁸ DHPS possesses a classic (β/α)₈ TIM barrel structure, and the *p*ABA binding site is at the edge of the barrel and is comprised of loop regions where the mutations that confer sulfonamide resistance are found.^{9, 10} In contrast, the other substrate of DHPS dihydropterin pyrophosphate (DHPP), binds in a deep structured pocket within the DHPS β-barrel. This pocket has a high degree of conservation between species and no resistance mutations have been reported in or adjacent to the pterin binding site. Thus, we believe that the pterin binding site is an attractive alternative target for the design of novel antimicrobial agents.

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Supporting Information

Additional figures as described in the text. This material is available free of charge via the internet at <http://pubs.acs.org>.

Currently, the principal screening methods for DHPS are either radiometric or use coupled colorimetric phosphate release assays.^{11–13} Although these assays do offer some advantages, they also suffer from a number of practical issues which pertain to their use for site specific high-throughput screening: The instability and limited availability of the DHPP pterin substrate, the undesirable use of radioactive materials, the need for co-incubation with pyrophosphatase and spectrometric interference in the colorimetric assays, and the lack of site specific inhibition information suitable for identifying the selective pterin-binding site inhibitors. In this paper, we report the design, development, and validation of a rapid, sensitive and site specific DHPS Fluorescence Polarization (FP) assay.¹⁴

A typical FP probe consists of a tracer derived from a high affinity ligand, a spacing linker and a fluorophore. Previously, we reported structural studies of several small pterin-based inhibitors of DHPS,^{15, 16} and the two most potent inhibitors **1** and **2** were selected as candidate tracers to develop the FP probes (Figure 1a). The co-crystal structure of DHPS bound to compound **2** was used as a guide to determine the optimal linker length between the tracer and the fluorophore. The carboxylate group of **2** points away from the pterin binding site and was judged to be a suitable linking position. From visual inspection, the distance from the solvent exposed surface to the carboxylate group was estimated to be approximately the length of two ethylene glycols (or aminoethanol). Based on this, three molecular probes with spacer lengths of 1, 2 and 3 ethylene glycols (or aminoethanol) were modeled into the cavity using Schrodinger Glide and our previously validated docking approach (see supplementary data, figure S1).¹⁷ From these studies, both the amide linked two and three glycol / aminoethanol long probes were shown to bind well. The three-unit spacer would be expected to improve solubility, and 2,2'-(ethane-1,2-diylbis(oxy))diethanamine was therefore selected as the linker to offset the poor aqueous solubility of the pterin analogs that has previously been encountered.¹⁸ Thus, **4** was chosen for synthesis and, using an analogs approach, probe **3** was also designed and selected for synthesis from **1** using a similar sized linker (Figure 1b).

Experimental section

Probe synthesis

Methanol, trifluoroacetic acid (TFA), dichloromethane, triethylamine (TEA), acetone, *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), formic acid and *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy) ethylcarbamate **6** were purchased from Sigma-Aldrich (St. Louis, MO). 2,5-Dioxopyrrolidin-1-yl 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate **9** was purchased from OChem, Inc. (Des Plaines, IL). *O*-(Benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (HBTU) was purchased from AK Scientific, Inc (Union City, CA). Compounds **2** and **5** were prepared as previously reported.^{16, 19} The reactions were monitored by thin layer chromatography (TLC) on pre-coated Merck 60 F254 silica gel plates and visualized by UV detection. The identity of final compounds was determined on a Waters Acquity UPLC MS C18 (H₂O + Formic → ACN + Formic) 5 minute gradient, PDA (215–400 nm), ELSD, Acquity SQD ESI Positive MS. High resolution mass spectra were determined on Waters Acquity UPLC C18 (H₂O → ACN gradient over 4 minutes) Xevo G2 Q-TOF ESI Negative, resolution mode, compound internally weighted to leucine enkephalin lock solution, calculated error <3 ppm. The purity of final compounds was determined by UPLC/UV/ELSD/MS (see supplementary data for UPLC/UV/ELSD/MS method). Both compounds **3** and **4** showed ELSD purity at 96% (see supplementary data, figure S3 and S4). Melting points were obtained on an OptiMelt Automated Melting Point System (Lambda Photometrics, UK) and were uncorrected. All ¹H and ¹³C spectra were recorded on a Bruker ULTRASHIELD™ 400 plus or Bruker 800/US². The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; coupling constants (*J*) are reported in hertz (Hz).

tert*-Butyl 2-(2-(2-((2-amino-5-nitroso-6-oxo-1,6-dihydropyrimidin-4-yl)amino)ethoxy)ethoxy)ethyl)carbamate **7*

A mixture of **5** (0.175 g, 0.938 mmol) and *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate **6** (0.233 g, 0.938 mmol) in methanol was heated at reflux for 20 h. After cooling the reaction mixture was filtered. To the filtrate was added 2.5 g silica gel and the solvent of filtrate was removed under reduced pressure to afford a dry plug, which was loaded on a silica gel column and eluted with chloroform/methanol (20:1). Fractions containing the product were pooled and evaporated to afford **7** as a brick red solid (0.21 g, 58%); R_f 0.4 (CHCl₃/CH₃OH=5:1); mp 166–168°C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.36 (s, 9H), 3.07 (q, *J* = 5.9 Hz, 2H), 3.40 (t, *J* = 6.1 Hz, 2H), 3.55 (t, *J* = 4.5 Hz, 8H), 6.78 (t, *J* = 5.6 Hz, 1H), 7.08 (s, 1H), 8.19 (s, 1H), 10.96 (s, 1H), 12.49 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 28.19 (s), 39.49, 68.17, 69.19, 69.44, 69.60, 77.53, 140.60, 152.21, 155.54, 156.37, 161.46; MS⁺ 387.31.

2-Amino-6-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-5-nitrosopyrimidin-4(3*H*)-one **8**

A solution of **7** (0.1 g, 0.259 mmol) in TFA/CH₂Cl₂ (v/v=1:1, 6ml) was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. Any remaining TFA was removed by repeated co-evaporation with methanol, ether and acetone to give **8** as trifluoroacetic acid salt a dark red solid (0.10 g, 97%); mp 200–204°C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.01–3.04 (m, 2H), 3.57–3.66 (m, 10H), 7.12 (s, 1H), 7.87 (s, 2H), 8.29 (s, 1H), 10.98 (s, 1H), 12.59 (s, 1H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 38.72, 66.73, 67.94, 69.59, 140.23, 152.44, 156.50, 158.57 (q, *J* = 128 Hz), 161.16; MS⁺ 287.26.

N*-(2-(2-(2-((2-Amino-5-nitroso-6-oxo-1,6-dihydropyrimidin-4-yl)amino)ethoxy)ethoxy)ethyl)-3,6-dihydroxy-3'-oxo-3'*H*,10*H*-spiro[anthracene-9,1'-isobenzofuran]-5'-carboxamide **3*

TEA (46.7 μl, 0.337 mmol) and 5-carboxyfluorescein *N*-hydroxysuccinimide ester **9** (0.044 g, 0.094 mmol) were added to a solution of **8** (0.03 g, 0.075 mmol) in acetone (4 mL). The reaction was stirred at room temperature for 4 h before being concentrated in vacuo. The crude residue purified by preparative HPLC using a Gemini 5μ C18 110A AXIA column (50 × 30.00 mm, 5 micron) and a gradient of 20–85% MeOH+4% THF/10 mM ammonium bicarbonate over 10 min period with the retention time of the product being 4.8 min. The respective fractions were pooled and concentrated to afford **3** as an orange solid (0.036 g, 75%); ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.47–3.59 (m, 12H), 6.42 (dd, *J* = 8.9, 2.2 Hz, 2H), 6.49 (d, *J* = 2.1 Hz, 2H), 6.62 (d, *J* = 8.9 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 1H), 8.12 (d, *J* = 8.0 Hz, 1H), 8.46 (d, *J* = 1.2 Hz, 1H), 8.88 (t, *J* = 5.5 Hz, 1H), 12.48 (s, 1H); ¹³C NMR (201 MHz, DMSO) δ 68.33, 68.86, 69.62 (d, *J* = 11.6 Hz), 102.41, 109.85, 116.15, 125.35, 126.14, 129.57, 131.80, 135.51, 140.72, 152.61, 153.74, 158.36, 163.96, 165.25, 166.20, 166.61, 168.41; HRMS *m/z* [*M*-H][−] calcd for C₃₁H₂₇N₆O₁₀: 643.5835, found: 643.1771.

tert*-Butyl 2-(2-(2-(2-(7-amino-1-methyl-4,5-dioxo-1,4,5,6-tetrahydropyridazino[3,4-*d*]pyrimidin-3-yl)propanamido)ethoxy)ethoxy)ethyl)carbamate **10*

To a solution of **2** (0.1 g, 0.377 mmol) and HBTU (0.157 g, 0.415 mmol) in DMF (5 mL), *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate **6** (0.112 g, 0.452 mmol) was added. The reaction mixture was stirred at room temperature for 3 h, diluted with water and extracted with dichloromethane twice. The organic layer was dried with sodium sulfate; the solvent was evaporated to give a white solid at room temperature. The residue was washed with anhydrous ether and filtered to give **10** as a white solid (0.155 g, 83%); R_f = 0.35 (CHCl₃/CH₃OH = 5:1); mp 230°C (dec); ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.22–1.24 (d, *J* = 7.2 Hz, 3H), 1.36 (s, 9H), 3.03–3.08 (m, 2H), 3.13–3.20 (m, 2H), 3.35–3.37 (m, 4H), 3.48 (s, 4H), 3.74 (s, 3H), 3.85–3.91 (q, *J* = 7.2 Hz, 1H), 6.76 (t, *J* = 5.5 Hz, 1H), 7.83 (t, *J* = 5.6

Hz, 1H), 7.95 (bs, 2H), 10.84 (bs, 1 H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 14.61, 28.19, 30.73, 35.74, 38.69, 41.00, 69.06 (d, J = 13.1 Hz), 69.48 (d, J = 13.6 Hz), 77.51, 155.54, 157.36, 162.25, 167.10, 171.95. MS $^+$ 496.33.

2-(7-Amino-1-methyl-4,5-dioxo-1,4,5,6-tetrahydropyridazino[3,4-*d*]pyrimidin-3-yl)-*N*-(2-(2-aminoethoxy)ethoxy)ethyl)propanamide 11

Compound **10** (0.3 g, 0.6 mmol) was added to a solution of TFA/ Cl_2Cl_2 (1:1 v/v). The reaction mixture was stirred at room temperature for 30 m. The solvent was removed under reduced pressure. Any remaining TFA was removed by repeated co-evaporation with diethyl ether to afford **11** as trifluoroacetic acid salt as a white solid (0.246 g, 80%): mp 160°C (dec); ^1H NMR (400 MHz, DMSO- d_6) δ 1.25–1.27 (d, J = 7.2 Hz, 3H), 1.36 (s, 9H), 3.00–3.03 (m, 2H), 3.09–3.27 (m, 2H), 3.39–3.43 (m, 4H), 3.53–3.60 (m, 2H), 3.64–3.72 (m, 2H), 3.78 (s, 3H), 3.93 (q, J = 7.2 Hz, 1H), 7.81 (t, J = 5.3 Hz, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 14.35, 41.19, 66.68, 68.91, 69.70, 100.69, 153.59, 155.46, 157.26, 158.11, 158.41, 160.19, 167.59, 171.70; MS $^+$ 396.26.

***N*-(2-(2-(2-(7-Amino-1-methyl-4,5-dioxo-1,4,5,6-tetrahydropyridazino[3,4-*d*]pyrimidin-3-yl)propanamido)ethoxy)ethoxy)ethyl)-3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamide 4**

A solution of **11** (0.050 g, 0.098 mmol), 2,5-dioxopyrrolidin-1-yl 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate **9** (0.058 g, 0.123 mmol) and TEA (61.2 μL , 0.442 mmol) in DMF (4 mL) was stirred at room temperature for 4h. The crude product was purified by preparative HPLC using a Gemini 5 μ C18 110A AXIA column (50 $\times$ 30.00 mm, 5 micron) and a gradient of 20–85% MeOH + 4% THF/10 mM ammonium bicarbonate over 10 min period. The retention time of the product was 4.8 min. The respective fractions were pooled and concentrated to afford the title compound **4** as an orange solid (0.050 g, 68%): ^1H NMR (400 MHz, DMSO- d_6) δ 1.21–1.23 (d, J = 8 Hz, 3H), 3.38–3.62 (m, 12H), 3.73 (s, 3H), 3.86–3.91 (q, J = 8 Hz, 1H), 6.46–6.49 (m, 2H), 6.56–6.62 (m, 4H), 7.33 (d, J = 8.0 Hz, 1H), 7.85 (t, J = 5.6 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.48 (bs, 1H), 8.89 (t, J = 5.2 Hz, 1H); ^{13}C NMR (201 MHz, DMSO) δ 14.52, 38.77, 40.93, 68.71, 69.03, 69.55, 100.72, 102.38, 109.86, 115.38, 124.71, 125.75, 129.46, 135.70, 153.29, 153.79, 156.64, 157.57, 161.36, 165.08, 167.28, 168.16, 171.96; HRMS m/z [M -H] $^-$ calcd for $\text{C}_{37}\text{H}_{34}\text{N}_7\text{O}_{11}$: 752.7100, found: 752.2316.

Protein preparation

Bacillus anthracis DHPS (*Ba*DHPS) was prepared as described previously.¹⁶

Probe Characterization - Isothermal Titration Calorimetry (ITC)

The purified *Ba*DHPS protein was dialyzed against 50 mM HEPES, 5mM MgCl_2 , pH 7.6. ITC titrations were performed with an Auto-iTC200 Isothermal Titration Calorimetry (MicroCal) in 40 mM HEPES, 4 mM MgCl_2 at pH 7.6 and 25 °C over nineteen 2 μL injections each of 500 μM ligand into 20 μM *Ba*DHPS. Data were analyzed using MicroCal Origin 7.0 software using a one-site binding model.

Probe Characterization – Fluorescence Polarization

The titration experiment was performed in triplicate with 6 nM of compounds **3** and **4** and 2-fold increasing concentrations of *Ba*DHPS (0.009 to 150 μM) in a 96-well plate with a final volume of 100 μL in 1X PBS buffer. Data were fit to a Langmuir isotherm:

$$FP = FP^0 + \Delta FP \cdot \left(\frac{I}{I + K_D/[DHPS]} \right)$$

To confirm pterin-pocket binding of **4**, inhibitor **2** was titrated (10 nM to 400 μ M) against 6 nM of **4** and 6 μ M of *Ba*DHPS in 1X PBS buffer to a final volume of 100 μ L in a black polystyrene 96-well plate (Costar, Corning Inc.). The data were fit to the equation derived from the definition of dissociation constant with the approximation of a negligible concentration of fluorescent probe described in the supplementary information.^{20, 21} All FP measurements were performed on a 2103 EnVision™ multilable reader (Perkin Elmer) with excitation and emission wavelength filters of 450 ± 8 nm and 535 ± 40 nm, respectively.

High Throughput Fluorescence Polarization Assay

In developing the FP assay for high throughput screening purposes, we followed the NIH NCGC guidelines (<http://www.ncgc.nih.gov/guidance/section5.html#practical-fluor-polar>). FP measurements were performed as described previously, but using PMT sensitivity set to low and 100 readings per well. Assays were performed in flat bottom, black polystyrene 384-well plates (3821 Costar, Corning Inc.) in 40 mM HEPES, 4 mM MgCl₂ at pH 7.6. The final assay volume was 20 μ L/well. All measurements were performed in triplicates except for Z' determination assays in which 12 replicates were used in each group. The typical final DMSO concentration in tests was 2% except for DMSO tolerance experiments in which varied DMSO concentrations were used as indicated.

To optimize the assay, equilibrium binding experiments of **4** to *Ba*DHPS were performed under the following conditions: each individual well in a 384-well assay plate contained 2.7 μ M **4** and increasing concentrations (from 0 to 16 μ M) of *Ba*DHPS protein in assay buffer. The plate was mixed on a slow moving shaker for the indicated times at 25°C and the polarization signals were measured at room temperature. To test the signal stability, the FP of a plate containing samples was measured at different times over a period of 18 hours. The assay equilibration time was determined by monitoring the FP signal at several time intervals as indicated. Between each reading, the plate was covered with a black lid to prevent photo-bleaching. For tracer specificity experiments, each individual well contained 2.7 μ M **4**, 8 μ M of *Ba*DHPS and DMSO; for competition experiments each individual well contained 2.7 μ M **4**, 8 μ M of *Ba*DHPS, 100 μ M of **2** and DMSO; each control well contained 2.7 μ M **4** and DMSO. The experiments were performed under conditions similar to those described above and the FP data were measured in triplicate. This experiment was repeated two more times on different days. To determine the effect of DMSO on the assay, DMSO tolerance experiments were performed under the above conditions with 2.7 μ M **4**, 8 μ M of *Ba*DHPS, and varying concentration of DMSO from 0.02% to 10% (v/v). To determine the assay robustness, Z'-factor statistic experiments were implemented in triplicate in which 2 groups of samples, the high signal group and low signal group, were involved. The high signal group represents samples with protein and tracer. The low signal group represents samples with protein, tracer, and ligand **2** at 100 μ M. This experiment was repeated two more times on different days. The Z' factor is calculated using the equation $Z' = 1 - (3\sigma^+ + 3\sigma^-) / (\text{Mean}^+ - \text{Mean}^-)$. Where σ^+ is the standard deviation of high signal group; σ^- is the standard deviation of low signal group; Mean^+ is the mean of high signal group and Mean^- is the mean of low signal group. All experimental data were analyzed using Microsoft® Excel (Microsoft Corporation, Redmond, WA) and Prism 4.0 (Graphpad Software Inc., San Diego, CA).

Assay Validation - Competitive Displacement of **4** by Known DHPS Inhibitors

Competitive displacement studies were performed with DHPS inhibitors **2**, **18**, **19**, **20**, pteric acid, SMX and SIA. Stocks of these inhibitors were prepared in DMSO at 10 mM and serially diluted in assay buffer. FP assay was performed under following conditions: Corning® black 384-well plate contained 2.7 μ M probe **4**, 8 μ M *Ba*DHPS protein, and each tested inhibitor at varying concentrations to a final volume of 20 μ L. The plate was kept shaking for 25 min at room temperature and the FP values were recorded. The measured FP values (mP) were plotted against the log₁₀ values of competitor concentration. Herein, the displacement data were empirically fit. In order to calculate accurate K_D values for hit compounds out of the HTS, where the approximation of a negligible concentration of fluorescent probe does not hold true due to experimental conditions, the exact analytical approach described by Wagner *et al.*^{20, 21} can be applied.

Results and discussion

The synthesis of FP probe **3** was achieved from 5-nitroso-6-methylpyrimidine **5**,¹⁹ which was conjugated with *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate **6** in refluxing methanol to give **7**.²² The Boc-protected **7** was converted to free terminal amine **8** by treatment with TFA. Coupling of **8** with 5-carboxyfluorescein *N*-succinimidyl ester **9** in the presence of TEA in DMF afforded the desired probe **3** in good yields.²³

The synthesis of FP probe **4** was synthesized from **2**. Coupling of **2** with **6** in the presence of HBTU in DMF provided amide **10**, which then was treated with TFA to afford the free amine **11**. Coupling of **11** with **9** in the presence of TEA in DMF afforded the second FP probe **4** in good yields.

Prior to establishing the FP assay, the equilibrium dissociation binding constants (K_D) of the two probes were measured using two independent methods, isothermal titration calorimetry (ITC) and FP. Using ITC, the K_D values were found to be 0.34 ± 0.043 μ M for compound **3** and 2.7 ± 0.64 μ M for compound **4** (Figure 2a, 2b). Using FP,^{24, 25} the K_D values were found to be in excellent agreement, 0.37 ± 0.1 μ M for compound **3** and 1.5 ± 0.1 μ M for compound **4** (Figure 2c, 2d). The affinities of both probes were found to be weaker than their corresponding parent inhibitors ($K_D = 0.124 \pm 0.017$ μ M for **2** using ITC). This was anticipated and is likely due to some loss of receptor binding interactions from the introduction of the linker groups and the bulky fluorescein fluorophore. Comparison of the two probes showed that **3** has an apparently higher binding affinity than **4** to *Ba*DHPS, but the ITC and the FP data for **3** both showed that binding was not at a 1:1 ratio whereas this was the case for **4** which has better FP signal. Further analysis by incubating **3** in 1% BSA showed that the FP absorbance of **3** was significantly reduced, which indicated that **3** is a non-specific protein binder. Consequently, **3** was dropped from further study and **4** was selected to develop the DHPS FP binding assays.

Finally, to confirm pterin-binding pocket binding, we monitored the displacement of **4** by its parent compound **2** at low probe concentrations using an FP competition experiment. The binding signal of **4** disappeared within a narrow concentration range of **2** (Figure 3), and curve fitting (see supplementary data) generated a K_D value for **2** of 0.24 ± 0.07 μ M, which agrees well with the value obtained by ITC (0.124 ± 0.017 μ M). It has already been confirmed by X-crystallography that **2** binds *Ba*DHPS at the pterin-binding site,¹⁶ and this experiment therefore confirms that the FP probe **4** is a competitive binder with respect to **2** and binds at the pterin-binding pocket in *Ba*DHPS as expected.

To develop the high throughput FP screening assay, it was necessary to determine the optimum protein concentration for competitive binding. Thus, **4** at its K_D (we chose the ITC

value of 2.7 μM) was titrated with varying concentrations of *Ba*DHPS (Figure 4) and the FP signal monitored. In the absence of protein, a low polarization value of 50 mP was observed, but with increasing concentrations of *Ba*DHPS protein, the FP signal progressively increased until it reached saturation. This revealed an assay window of up to 200 ΔmP which is a robust signal window for a FP assay. By examining the response curve (Figure 4), DHPS at 8 μM was selected for further assay development because this concentration yields an acceptable assay window of $\Delta\text{mP} \sim 150$ mP. This experiment was also used to evaluate the stability of the FP signal, which becomes an important parameter in the high-throughput screening of large number of compounds over long periods of time.²⁶ To this end, the probe was incubated with *Ba*DHPS for a period of 18 hours within which the FP signal of the plate was read several times. The binding curves showed that the FP signal is very stable with little noticeable difference. In addition, incubation of the probe with protein reached signal saturation in a very short time (~ 20 min), which is an added advantage (Figure 4).

DMSO is routinely used to dissolve or dilute compounds for screening assays; therefore the effect of various DMSO concentrations on the FP signal was studied. Specifically, **4** was incubated with *Ba*DHPS in the presence of varying DMSO concentrations up to 10% and the variation in FP signal was monitored (see supplementary data, figure S2). This experiment showed that the FP binding assay tolerated DMSO well, up to a concentration of 10%, with less than 10 % reduction of the FP signal which remained stable for 18 hours (higher DMSO concentrations were not tested because the DMSO concentration will not exceed 10% in a typical assay).

To evaluate the performance of the DHPS FP binding assay, the Z' value was calculated for the assay carried out in a 384 well plate format.²⁷ In general, Z' values between 0.5 and 1.0 are considered acceptable. Here, the Z' values for the *Ba*DHPS FP binding assay obtained from three independent experiments were 0.87, 0.87 and 0.86, respectively (see supplementary data, figure S3), and these values showed that the assay is highly reproducible. Furthermore, the FP assay with **4** was also tested against *Staphylococcus aureus* DHPS (*Sa*DHPS) using similar assay conditions, and this also performed remarkably well with a Z' value at 0.84. This confirms that the assay is suitable for future high-throughput screening against multiple DHPS enzymes.

Finally, competitive displacement assays were performed with various known DHPS inhibitors to test the robustness of the developed FP assay and to validate its utility for detecting specific inhibitors that target the pterin-binding pocket. In an initial competition binding experiment in which *Ba*DHPS, probe **4** and its parent inhibitor **2** were co-incubated under high throughput assay optimized conditions, the binding signal of **4** was completely abolished by an excess of **2** and the FP signal was almost identical to that for probe **4** in the absence of protein. The results were stable over a three-day period (Figure 5) and are consistent with the titration experiment shown in Figure 3. Seven inhibitors were then tested: **2**, **18** (an analog of **2**) and **19** are DHPS inhibitors that are known to target the pterin-binding pocket,¹⁶ sulfamethoxazole (SMX) and sulfanilamide (SIA) are two sulfonamides that bind the *p*ABA binding pocket, and **20** and pteric acid are two DHPS inhibitors that engage both the pterin- and *p*ABA-binding sites simultaneously. The competition experiment (Figure 6) showed that all the compounds known to bind the pterin-binding site on DHPS (**2**, **18**, **19**, **20** and pteric acid) are able to compete off the FP probe **4** and are identified as hits in the current assay. However, SMX and SIA, which bind to the *p*ABA sub-site are not able to compete off the FP probe **4** and thus would not be identified as hits since the assay described herein specifically identifies compounds that bind to the pterin sub-site.

In summary, two pterin-based fluorophores **3** and **4** were designed, synthesized, and evaluated as probes for the development of a FP assay for screening against *Ba*DHPS and *Sa*DHPS. The FP assay described herein, in which **4** is used as probe, is rapid, accurate, robust, and reproducible as evidenced by an excellent Z' factor. Furthermore, it has great tolerance with respect to the typical concentrations of DMSO used in small molecule screening and is suitable for identifying DHPS inhibitors that bind to the pterin sub-site in a high throughput format. Finally, **4** can be further used to subsequently derive K_D values for the pterin pocket inhibitors identified in the high throughput FP assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DHPS	Dihydropteroate synthase
FP	fluorescence polarization
pABA	<i>p</i> -amino benzoic acid
DHPP	dihydropterin pyrophosphate
ITC	isothermal titration calorimetry
<i>Ba</i>DHPS	<i>Bacillus anthracis</i> DHPS
<i>Sa</i>DHPS	<i>Staphylococcus aureus</i> DHPS
SMX	Sulfamethoxazole
SIA	Sulfanilamide

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Figure 1.

a) Structures of pterin-based DHPS inhibitors **1** and **2**. b) Structures of the designed FP probes **3** and **4**

Figure 2.

Measuring direct binding of compounds **3** and **4** to *Bacillus anthracis* DHPS (BaDHPS). a) ITC titration of 500 μM of **3** into a 20 μM BaDHPS solution. Binding site $N = 1.59 \pm 0.0108$; ΔH is -5.9 ± 0.059 kcal/mol; ΔS is 0.0982 kcal/mol; K_D is 0.34 ± 0.043 μM . b) ITC titration of 500 μM of **4** into a 20 μM BaDHPS solution. Binding site $N = 1.09 \pm 0.0464$; ΔH is -2.8 ± 0.159 kcal/mol; ΔS is 0.16 kcal/mol; K_D is 2.7 ± 0.64 μM . c) Direct binding of **3** to BaDHPS by FP (average of 3 independent experiments). Error bars represent standard error of the mean; K_D is 0.37 $\mu\text{M} \pm 0.1$. d) Direct binding of **4** to BaDHPS by FP

(average of 3 independent experiments). Error bars represent standard error of the mean; K_D is $1.5 \pm 0.1 \mu\text{M}$.

Figure 3.

FP competition titration of non-fluorescent compound **2** (10 nM to 400 μM) against compound **4** (6 nM), and 6 μM BaDHPS (average of 3 independent experiments). $K_D = 0.24 \pm 0.07 \mu\text{M}$.

Figure 4.

Titration of **4** (2.7 μ M) with *Ba*DHPS monitored by FP. Increasing concentrations of *Ba*DHPS (0–16 μ M) was incubated with **4** at 25 $^{\circ}$ C, and the response measured at 20 min, 80 min, and 18 h.

Figure 5. Tracer specificity test. ■ 2.7 μ M of 4 with 8 μ M of BaDHPS in buffer; ■ 2.7 μ M of 4, 8 μ M of BaDHPS and 100 μ M of 2 in buffer; ■ 2.7 μ M of 4 only in buffer

Figure 6.

a) Structure of small molecule inhibitors evaluated in FP competition experiment; b) Competitive displacement of **4** from *Ba*DHPS by various DHPS inhibitors.

Scheme 1.

Synthesis of FP probe **3**. *Reagents and conditions*: a) *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate **6**, CH₃OH, reflux, 20 h, 58%; b) TFA, 97%; c) 5-carboxyfluorescein *N*-succinimidyl ester **9**, DMF, TEA, 75%.

Scheme 2.

Synthesis of probe 4. *Reagents and conditions:* a) *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate 6, HBTU, DMF, rt, 3 h, 83%; b) TFA, 80%; c) 5-carboxyfluorescein *N*-succinimidyl ester 9, DMF, TEA, 68%.