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Discovery and Characterization of Halogenated Xanthene Inhibitors of DUSP5 as Potential Photodynamic Therapeutics

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Robert Bongard: Designed experiment, interpreted data, and assembled figures and manuscript preparation.

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Daniel S. Sem: Key role in guiding group members in the characterization of DUSP5 enzyme kinetics and activity; aided in all aspects of project direction, compiled, prepared and edited the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Abstract

Dual specific phosphatases (DUSPs) are an important class of mitogen-activated protein kinase (MAPK) regulators, and are drug targets for treating vascular diseases. Previously we had shown that DUSP5 plays a role in embryonic vertebrate vascular patterning. Herein, we screened a library of FDA-approved drugs and related compounds, using a *para*-nitrophenylphosphate substrate (*p*NPP)-based assay. This assay identified merbromin (also known as mercurochrome) as targeting DUSP5; and, we subsequently identified xanthene-ring based merbromin analogs eosin Y, erythrosin B, and rose bengal, all of which inhibit DUSP5 *in vitro*. Inhibition was time-dependent for merbromin, eosin Y, 2',7'-dibromofluorescein, and 2',7'-dichlorofluorescein, with enzyme inhibition increasing over time. Reaction progress curve data fit best to a slow-binding model of irreversible enzyme inactivation. Potency of the time-dependent compounds, except for 2',7'-dichlorofluorescein, was diminished when dithiothreitol (DTT) was present, suggesting thiol reactivity. Two additional merbromin analogs, erythrosin B and rose bengal also inhibit DUSP5, but have the therapeutic advantage of being less sensitive to DTT and exhibiting little time dependence for inhibition. Inhibition potency is correlated with the xanthene dye's LUMO energy, which affects ability to form light-activated radical anions, a likely active inhibitor form. Consistent with this hypothesis, rose bengal inhibition is light-dependent and demonstrates the expected red shifted spectrum upon binding to DUSP5, with a K_d of 690 nM. These studies provide a mechanistic foundation for further development of xanthene dyes for treating vascular diseases that respond to DUSP5 inhibition, with the following relative potencies: rose bengal > merbromin > erythrosin B > eosin Y.

Graphical abstract

Keywords

DUSP5; pERK; eosin Y; xanthene dyes; merbromin; rose bengal; enzyme kinetics

INTRODUCTION

Dual specificity phosphatases (DUSPs) are a unique class of mitogen-activated kinase phosphatases (MKPs) that remove phosphate groups from nuclear-localized MAP kinases (type I), cytoplasmic-localized MAP kinases (type II) or function in both nucleus and cytoplasmic compartment (type III) ¹. Our laboratory is interested in DUSP5, which is a typical type I nuclear inducible MKPs; and, we have previously shown that DUSP5 is important for embryonic vascular patterning ². Recently ^{3–5} loss and gain of expression of *Dusp5* in murine models has been associated with phenotypic changes in both immune and cancer biology systems. *Dusp5* knockout (KO) mice appear healthy, and display no overt phenotype, indicating that *Dusp5* is dispensable for embryonic development. Holmes *et al* reported that *Dusp5* KO mice showed increased function and survival of eosinophils, which play an important role in the immune system's ability to clear parasitic infections ³. Others have reported increased sensitivity to skin cancer in their *Dusp5* murine model ⁵. Collectively, these studies implicate an important function for DUSP5 in mammalian biology, and a possible role of DUSP5 as a drug target.

Our interest in DUSP5 relates to its potential role in diseases related to the vasculature. Previously, we identified a clinically relevant serine to proline mutation (S147P) in *Dusp5* that is associated with vascular anomalies ², a disorder of vascular development. Of all the DUSPs, DUSP5 is unique in that its substrate specificity is almost exclusive to extracellular-regulated kinase (ERK). DUSP5 dephosphorylates pERK1/2 in the nucleus, and shuts down proliferative signals. The S147P mutation has been shown previously by our group to interfere with the dephosphorylating activity of DUSP5 protein ⁶, and makes the protein hypoactive towards pERK1/2. Selectivity of DUSP5 toward pERK2 is provided by the structural composition of DUSP5 that consists of two globular domains—ERK-binding domain (EBD) and phosphatase domain (PD)—connected by a ~40 amino acid-long linker, which also contributes to the selectivity toward pERK ⁶. Additionally, our previous molecular dynamics simulation work ⁷ indicates a secondary binding site as a critical regulator of the phosphatase activity of DUSP5.

DUSP5's function is increasingly being recognized as context-dependent. In melanoma and colorectal neoplasms ^{8,9}, DUSP5 is associated as a promoter of growth while in prostate and gastric neoplasms ^{10,11} it is associated as a suppressor of growth. Similarly, DUSP5's role in the nucleus and cytoplasm of cells as it relates to ERK is contextual. In the nucleus, DUSP5 dephosphorylates pERK and turns off ERK signaling ¹², while in the cytoplasm it facilitates paradoxical ERK signaling by relieving RAF-mediated inhibition of ERK ¹³. Because DUSP5's function is versatile, developing activators and inhibitors of DUSP5 are of equal importance.

With this objective in mind, in this study, we screened a library of FDA-approved drugs and related compounds for DUSP5 inhibitors with the goal of rapidly repurposing these

inhibitors for vascular anomalies treatment. Our study identified merbromin (also called mercurochrome), a topical antiseptic, and subsequently merbromin analogs eosin Y, 2',7'-dibromofluorescein, 2',7'-dichlorofluorescein, erythrosin B, and rose bengal (Fig. 1), each of which inhibits DUSP5 activity *in vitro*. All compounds are xanthene ring dyes, and some are photoreactive and are known to form charge-transfer complexes with proteins (see Discussion section). Intriguingly, eosin Y has already been used topically in select ulcerative hemangioma patients in the clinic, and showed efficacy ¹⁴. Likewise, rose bengal recently completed Phase 2 clinical trials as a light activated (photodynamic) therapeutic agent for treating melanoma cancer ¹⁵. The studies presented herein characterize DUSP5 inhibitors, including rose bengal, as potential target-based laser-induced photoactivatable compounds as plausible treatment options for vascular anomalies that are on the surface of the skin.

METHODS

Drug Library Enzyme Assay Screen

The Prestwick Chemical Library, which contains 1,280 small molecules/drugs that have been approved by various regulatory agencies, and their analogs, were obtained from Prestwick Chemical, Inc. (Illkirch-Graffenstaden, France) and screened as inhibitors of DUSP5 (Supplementary Fig. S1). Daughter 96 well plates were generated from diluting the master 96 well plates (10 mM) to 4 mM in *p*NPP buffer (100 mM NaCl, 5 mM MgCl₂, 0.5% Triton-X 100, 30 mM Tris-HCl pH 7.5, 1 mM DTT, H₂O), which were further diluted to 100 μ M working plates. Dilutions were performed using the iLink Pro program (Caliper Lifesciences, Hopkinton MA), which controlled a robotics system comprised of a Sciclone ALH3000 workstation (Caliper Lifesciences), a Powerwave XS plate reader (Biotek Instruments Inc, Winooski VT), a Twister II microplate handler (Biotek), and a StoreX IC incubator (Liconic US Inc, Woburn MA). Reactions in each well contained 100 μ L of the 100 μ M drug to be tested. Control wells contained either no drug (100 μ L *p*NPP buffer), blank wells (150 μ L *p*NPP buffer only), or positive control (100 μ L of 400 μ M Na₃VO₄). 50 μ L of GST-DUSP5 (4 μ M) was added to all drug containing, no drug, and positive control wells and allowed to incubate for 5 minutes. After incubation 50 μ L of *p*NPP (20 mM) was added to the same wells to give final concentrations of 50 μ M drug, 1 μ M GST-DUSP5, and 5 mM *p*NPP. Data from wells containing drug were normalized to no drug wells from the same plate to determine the percent of DUSP5 activity.

In vitro ERK Dephosphorylation Western Blot Assay and IC₅₀ Determination

GST-DUSP5 purified protein was generated using previously published methods ⁶. The protein was diluted in phospho-ERK buffer (30 mM Tris-HCl pH 7.0, 75 mM NaCl, 0.67 mM EDTA, 1 mM DTT, H₂O) to a concentration of 1.5-3.0 nM, depending on the purity. Active ERK2 (R&D Systems, Minneapolis MN) and the drugs to be tested were also diluted in this buffer with an initial concentration of 30 nM for ERK2 and serial dilutions for the drugs. 5 μ L each of GST-DUSP5 and diluted drug concentrations were incubated for 5 mins after which 5 μ L of 30 nM ERK2 was added and allowed to incubate for 20 mins. After this time 15 μ L SDS-Loading buffer was added to each reaction. Samples were boiled for 5 mins, loaded into lanes of 12% Mini-Protein TGX gels (Bio-Rad Laboratories Inc, Hercules CA), and ran at 120V until they had migrated the appropriate distance through the gel

(Supplementary Fig. S1c). Protein samples were then transferred to PVDF Western Blotting Membranes (Roche Diagnostics, Indianapolis IN) at 90V for 1 h. Membranes were treated and utilized in the iBind Flex Western Device (Thermo Fisher Scientific, Waltham MA) according to manufacturer protocols. Membranes were probed for total and phospho-ERK using rabbit anti-human p44/42 MAPK and mouse anti-human phospho-p44/42 MAPK primary antibodies and HRP-linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling Technology Inc, Danvers MA). Images were developed using a FluorChem HD2 imager (Bio-Techne, Minneapolis MN) after application of SuperSignal West Femto and West Pico chemiluminescent substrate (Thermo Fisher Scientific).

IC₅₀ Calculation

Densitometry analysis of western blot images was performed using ImageJ software. Values obtained were used in GraphPad Prism 6 software to calculate a non-linear regression (curve fit) to equation 1;

$$y = \min + \left(\frac{(\max - \min)}{1 + 10^{(x - \log IC_{50})}} \right) \quad (\text{equation 1})$$

with x equal to the merbromin or eosin Y concentration, y equal to the relative densitometry value at a given compound concentration, $\max$ equal to the normalized densitometry value of the p ERK without DUSP5 sample, and $\min$ equal to the normalized densitometry value of the sample containing p ERK and DUSP5 without added compound. Sigmoidal dose-response curves with merbromin and eosin Y were fitted to using equation 1 which generated IC₅₀ estimates for both compounds.

DUSP5 Phosphatase Domain Protein Synthesis and pNPP Assay

The *Dusp5* phosphatase domain gene was synthesized by Blue Heron (Bothell, WA) and the protein expressed and purified as previously described¹⁶. To measure the phosphatase activity of wild type phosphatase domain (DUSP5 PD) enzyme, and the inhibitory capacity of selected compounds, an *in vitro* phosphatase assay was utilized¹⁶ (Figs. 2–3; Supplementary Figs. S2-S3). Briefly, assays without and with inhibitors were performed in Greiner 96-well clear bottom plates with a total assay volume of 200 μ L. Assay buffer contained 100 mM Tris, 100 mM NaCl, 5 mM MgCl₂·6H₂O without or with 1 mM dithiothreitol (DTT) at pH 7.5. p -nitrophenol phosphate (p NPP, Sigma Aldrich) at 5 mM, a concentration near the established K_m , was used as the substrate. DUSP5 PD-mediated hydrolysis of p NPP to p -nitrophenolate, a chromogenic product which absorbs at 405 nm with a molar extinction coefficient of 18,000 M⁻¹ cm⁻¹, was monitored over time using a Spectramax M5 microplate reader (Molecular Devices). Wells that contained assay buffer with p NPP, without and with DTT were used as blanks, and wells containing the same buffer as above with the addition of DUSP5 PD enzyme were used as negative controls. Stock solutions (2.5 mM to 15 mM) of the study compounds merbromin, eosin Y disodium salt, fluorescein sodium salt, erythrosin B, rose bengal (Sigma Aldrich), 2',7'-dibromofluorescein (synthesized in-house), and 2',7'-dichlorofluorescein (synthesized in-house) were prepared in either double deionized water or DMSO. Serial dilutions of each stock solution were prepared to generate a range of inhibitor concentrations for screening purposes. 4 μ L of the

serially diluted inhibitors were added into wells containing 192 μL assay buffer, resulting in range of inhibitor concentrations of 0.3 μM to 50 μM for merbromin, 0.3 μM to 100 μM for eosin Y, 1.0 μM to 300 μM for 2',7'-dibromofluorescein, 1.0 μM to 300 μM for 2',7'-dichlorofluorescein, 0.3 μM to 30 μM for erythrosin B, 0.04 μM to 20 μM for rose bengal, and 1.0 μM to 300 μM for fluorescein. Appropriate vehicles in the place of inhibitor compound were added to the blank and negative control wells. The reaction was initiated by dispensing 4 μL of a 25 μM enzyme stock into each of the wells except the blanks, in which case 4 μL enzyme diluent buffer was added. The plate was immediately placed into the plate reader, shaken, and absorbance at 405 nm recorded at 25°C every 30 seconds for ten minutes (initial assay). Sample absorbance was recorded in a similar manner following 30 and 60 minute incubations of the enzyme with the respective compounds in the dark. To further investigate possible time-dependent DUSP5 PD inhibition by the study compounds, additional dose-response experiments were performed under the similar conditions. Activity assays were performed uninterrupted for 120 min and absorbance recorded at one min intervals for the duration of the assay. Absorbance values were converted to *para*-nitrophenolate concentration using the product extinction coefficient and a micro well path length of 0.545 cm. Three or four trials of three replicate wells were run for each condition at each inhibitor concentration. A new assay plate was used for each trial. Replicate well data in the same plate was averaged to yield a single data point at each inhibitor concentration for each trial.

Solution-state Enzymatic Assays and IC_{50} Calculations

Slope values for the change in absorbance over the initial ten min assay period for all negative control wells were averaged following blank subtraction, and the averaged value considered representative of full enzymatic activity. Slope values of replicate wells containing inhibitor compounds were also averaged, and the fractional activity calculated by dividing the averaged slope value at each inhibitor concentration by the averaged negative control slope. Percent activity was plotted versus the log inhibitor concentration and the data fitted to a non-linear least squares variable slope model (GraphPad Prism 6) using equation 2;

$$y = \text{bottom} + \left(\frac{(\text{top} - \text{bottom})}{1 + 10^{(\log \text{IC}_{50} - x) * \text{Hill slope}}} \right) \quad (\text{equation 2})$$

with y as the percent enzyme activity at a given inhibitor concentration relative to a negative control, *top* and *bottom* are plateau enzyme activities of the uninhibited and fully inhibited enzyme, constrained to 100 and 0 percent, respectively (Figs. 2–3; Supplementary Figs. S2–S3). IC_{50} values were determined in the same manner for experiments in which the enzyme had been incubated with inhibitors for 30 or 60 min before enzyme activity was assayed. Inhibition values are summarized in Table 1.

Kinetic Assessment of Enzyme Inhibition for Compounds Exhibiting Non-linear Reaction Progress Curves

Reaction progress curves of DUSP5 PD in the presence of merbromin, eosin Y, 2',7'-dibromofluorescein, and to a lesser extent, 2',7'-dichlorofluorescein displayed a non-linear

velocity profile, with IC₅₀ curves that shifted left with incubation time, suggesting a time-dependent inhibition mechanism (Table 1). Non-linear enzyme activity progress curves with *p*NPP as the substrate were monitored over a two-hour period over a range of inhibitor concentrations and the data at each concentration fitted to the equation for irreversible enzyme inactivation (Figs. 5, 6; Supplementary Figs. S4-S10)¹⁷;

$$[P] = \frac{v_i}{k_{obs}} \left[1 - \exp(-k_{obs} * t) \right] \quad (\text{equation 3})$$

with *P* as the *para*-nitrophenolate concentration, *t* as time, *v_i* the initial reaction velocity and *k_{obs}* the apparent first order rate constant for the formation of the inactive enzyme-inhibitor complex. The resulting *k_{obs}* values obtained at each inhibitor concentration were then replotted versus inhibitor concentration and yielded relationships that were either linear or hyperbolic in nature. Linear data were fitted with a linear least-squares model, and the hyperbolic data fitted to equation 4 (Figs. 5, 6; Supplementary Figs. S4-S10)¹⁷;

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]} \quad (\text{equation 4})$$

where *k_{inact}* is a first order rate constant describing the maximum rate of covalent bond formation at an infinite concentration of inhibitor, *I* is the inhibitor concentration, and *K_I* is the dissociation constant describing the concentration of inhibitor required for half the maximal rate of covalent bond formation. The linear and hyperbolic relationships describe two different mechanisms of enzyme inhibition depicted in Scheme 1 below:

The linear, non-saturating relationship of *k_{obs}* as a function of *I* describes a covalent, one-step model of irreversible inhibition reflecting a non-specific reaction between the inhibitor and enzyme (Mechanism 1, Scheme 1). The slow onset of inhibition results from the inherent slow rates of compound association to form the covalent complex. The apparent compound potency, described by the second order inactivation rate constant relationship *k_{inact}*/*K_I*, having second order rate constant units of M⁻¹s⁻¹, was estimated from the slope of the linear least-squares regression line fit to the data. The hyperbolic relationship of the pseudo first order rate constant and inhibitor compound concentration describes a two-step inhibition mechanism, (Mechanism 2, Scheme 1) including a reversible binding and unbinding of the inhibitor to the enzyme, forming an intermediate enzyme inhibitor complex, followed by an irreversible transformation of the intermediate enzyme inhibitor complex into an inactive form, denoted by E-I (Scheme 1). Inhibition values are summarized in Table 2.

Kinetic Assessment of Enzyme Inhibition for Compounds Exhibiting Linear Reaction Progress Curves

Linear reaction progress curves over a range of inhibitor concentrations generally suggest rapid reversible binding and dissociation of the inhibitor from its target enzyme, with no shift in the activity versus inhibitor concentration (IC₅₀) curves with incubation time. Under such circumstances, the mechanism of inhibition can usually be analyzed by classical methods. In the present study, erythrosin B displayed only modest time-dependent DUSP5

PD inhibition and little sensitivity to DTT. DUSP5 PD enzyme activity curves over a range of substrate and erythrosin B concentrations in the presence of 1 mM DTT were fitted globally using GraphPad Prism 6 to equation 5 for steady-state competitive inhibition;

$$v = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S]} \quad (\text{equation 5})$$

where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, $[S]$ is the p NPP concentration, I is the erythrosin B concentration, and K_i the inhibition constant. The parameters V_{max} , K_m , and K_i are shared, resulting in one best-fit value for the complete data set. DUSP5 PD reaction progress curves in the presence of rose bengal were also linear, exhibiting no time-dependent inhibition or DTT sensitivity. Fitted results are summarized in Supplementary Table S2.

Incandescent bulb kinetic studies—Rose bengal has been reported to be a photo-sensitizing reagent; thus, we investigated the effects rose bengal of DUSP5 PD activity following incubating the enzyme with various rose bengal concentrations under conditions in which light exposure was limited, or the mixture was illuminated with an incandescent light source (Fig. S11, S12). DUSP5 PD enzyme activity was monitored in assay buffer containing 100 mM Tris, pH 7.5, 100 mM NaCh, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 1 mM DTT with p NPP as the substrate. 150 μL of stock DUSP5 PD protein (2.45 mg/mL) was added to 20 mL assay buffer which had been cooled to 4°C, to a final enzyme concentration of 1 μM . A 1 mM stock concentration of rose bengal was prepared in double deionized sterile water and stored protected from light. Stock concentrations of p NPP at 60, 180, and 540 mM were prepared in double deionized sterile water. A 6.5 mL assay buffer volume containing DUSP5 PD was removed from the initial 20 mL volume and pipetted into a 16 mm $\times$ 25 mm borosilicate glass tube having a 1 mm wall thickness (VWR cat. no. 47729-578), and the mouth of the tube parafilm. To the remaining 13.5 mL assay buffer containing enzyme, 13.5, 33.75, or 47.25 μL stock rose bengal was added which resulted in final rose Bengal concentrations of 1, 2.5, and 3.5 μM , respectively. Two additional tubes were prepared with the addition of 6.5 mL buffer containing protein and rose bengal which were sealed with parafilm, and one tube wrapped with aluminum foil (shielded) to avoid light exposure. The three tubes were placed in a test tube rack and placed inside a chromatography refrigerator (VWR) maintained at 4°C. The rack was positioned such that the face of the two tubes without foil and the shielded tubes were located 15 and 15.3 cm, respectively, from the center of a 100 watt incandescent bulb rated at 1,230 lumens, which was secured to a stand inside the refrigeration unit. Illuminance at the tube faces was monitored with an illuminance meter (Sper Scientific, model 840010C) and averaged between 3560 and 3625 lux for the shielded and unshielded tubes, respectively. A lux value of zero was determined when the sensor was placed inside a tube wrapped with foil and located 15 cm from the light source. The tubes were illuminated for a 15 minute period after which the light was switched off and the previously unshielded tubes were wrapped in foil and removed from the refrigerator. The effect of the 15 minute light exposure on sample temperature was monitored in replicate experiments (Supplementary Fig. S13) using a TRACEABLE® thermocouple (VWR Scientific). Following the illumination period, the two unshielded

samples were wrapped with aluminum foil, and all tubes incubated in the dark at 25° C for 15 minutes, allowing samples to warm to room temperature. DUSP5 PD enzyme activity was determined with the addition 1.9 mL volumes of each sample to quartz cuvettes. Activity assays were performed at 25°C and initiated with the addition of 100 μ L *p*NPP stock solutions to the cuvettes, which resulted in final *p*NPP assay concentrations of 3, 9, and 27 mM. Absorbance was recorded at 405 nm in 30 second intervals for 15 minutes using a Genesys UV-VIS spectrophotometer (Thermo Scientific). Assay buffer without additions was used as the blank and assay buffer samples without enzyme but containing 3, 9, and 27 mM *p*NPP were used for background subtraction. The rate of *p*NPP hydrolysis product (para-nitrophenolate) generation was determined using an extinction coefficient of 18,000 $\text{M}^{-1}\text{cm}^{-1}$. Initial velocities were calculated from linear least squares fits of the change in sample para-nitrophenolate concentration with time and reported in units of $\mu\text{M}\cdot\text{min}^{-1}$. Initial velocities were plotted against *p*NPP concentration for each of the rose bengal concentrations and the resulting dose response curves fitted globally to two noncompetitive inhibition models (Fig. S11, S12). One model was the noncompetitive inhibition model in the GraphPad Prism 6 software, with a single K_i (Table S2);

$$v = \frac{\left(\frac{V_{max}}{1 + \frac{[I]}{K_i}} \right) * [S]}{K_m + [S]} \quad (\text{equation 6})$$

where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, $[S]$ is the *p*NPP concentration, I is the rose bengal concentration, and K_i the inhibition constant. The second model was for noncompetitive inhibition, and contained two K_i values (a slope and an intercept K_i) (Table S2);

$$v = \frac{V_{max} * [S]}{\left(1 + \frac{[I]}{K_{is}} \right) * K_m + \left(1 + \frac{[I]}{K_{ii}} \right) * [S]} \quad (\text{equation 7})$$

where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, $[S]$ is the *p*NPP concentration, I is the rose bengal concentration, K_{is} is the slope inhibition constant of rose bengal (with respect to the free enzyme,) and K_{ii} the intercept inhibition constant (with respect to the enzyme-substrate complex).

Headspace gas sparging kinetic studies—To investigate the role of oxygen on rose bengal-mediated photo-inactivation of DUSP5 PD enzyme activity (Supplementary Fig. S15), 4 mL assay buffer samples in borosilicate tubes containing 1 μM DUSP5 PD without or with 1 μM rose Bengal were prepared shielded from light, capped with rubber stoppers and placed on ice. Sample tube headspace was accessed with the insertion of a 21 gauge 1½ inch needle through the stopper. Ultrapure compressed air, nitrogen, or oxygen was introduced to the headspace at rates of 2.5 liters per minute. Gas exhaust was accomplished with an 18 gauge needle inserted through the stopper. Stoppered, shielded tubes containing 4 mL assay buffer with 1 μM enzyme but without rose bengal, and with headspace room air gas were used as controls. Samples were sparged in the dark for 15 minutes after which the

sample ports were occluded. The sample tubes were then placed in a chromatography refrigerator, the foil removed, then illuminated for 15 minutes at 3500 to 3625 lux using a 100 watt incandescent light, after which the tubes were placed in the dark and warmed to room temperature. DUSP5 PD enzyme activity following illumination was monitored using a 96 well plate format. Wells were filled with 190 μL of the gassed and control samples in replicates of eight, and the activity assay initiated with the addition of 10 μL 180 mM *p*NPP, which resulted in a final *p*NPP assay concentration of 9 mM. A 200 μL volume of assay buffer without additions was used as the blank, and 190 μL assay buffer without enzyme but with *p*NPP was used for background subtraction. Absorbance was recorded at 405 nm in 30 second intervals for a period of 10 minutes at 25°C using a SpectraMax M5 plate reader. Absorbance data were transformed to para-nitrophenolate concentration, accounting for the micro well volume light path length. Initial velocities were calculated from linear least squares fits of the increase in sample para-nitrophenolate concentration with time data and reported in units of $\mu\text{M}\cdot\text{min}^{-1}$.

LED Illumination Kinetic Studies—Additional illumination experiments with rose bengal were performed with a 555 nm LED light source (Thorlabs LED555L) (Supplementary Table S2; Supplementary Fig. S14). The reported peak LED emission wavelength of 555 nm and 40 nm bandwidth overlapped well with the rose bengal absorption spectrum. The LED was connected to a T-cube LED driver (Thorlabs LEDD1B) which allowed for control of the current from 0 to 1200 mA, and powered with a 15 V, 2.4 A power supply (Thorlabs KPS10). The manufacturer's specifications for the LED included a suggested a continuous operating current of 20 mA, an optical output power at 50 mA of 1 mW, and a viewing angle of 20°. Assay buffer samples of 4 mL containing 1 μM enzyme and 0, 1, 2, and 3.5 μM rose bengal were prepared in borosilicate glass tubes at 20°C under lux conditions of less than 1. Sample tubes were placed 3 cm from the front of the LED lens and illuminated at 20°C for 30 minutes. A continuous operating current of 22 mA was utilized and set with the LED driver. Using this current, the manufacturer's supplied output power, current, and forward voltage specifications suggested the generation of 1.2 mW of optical output power, resulting in a calculated light power density value of 0.3 mW/cm² at the face of the sample tube positioned 3 cm from the LED. An illuminance value of 825 lux was recorded at a 3 cm distance from the LED. Matched sample tubes were wrapped in foil and stored in a cabinet to ensure the absence of light exposure. Temperature measurements of replicate samples with a thermocouple showed no change over the time course of LED illumination. Following the 30 minute treatments, enzyme activity was assayed in a 96 well plate format using *p*NPP as the substrate. Sixteen wells received 190 μL of illuminated sample and sixteen wells received 190 μL of light shielded sample, both in a 4 $\times$ 4 format. Reactions were initiated with the addition of 10 μL *p*NPP vehicle or 10 μL of 60, 180, and 540 mM stock *p*NPP, each in replicates of four, resulting in final *p*NPP assay concentrations of 0, 3, 9, and 27 mM. Assay plate preparation was performed under conditions of less than 1 lux. Assay buffer samples without additions were used as blanks and assay buffer without enzyme, but with the *p*NPP additions, were used for background subtraction. The plate was assayed at 25°C using a plate reader. Absorbance was recorded at 405 nm in 30 second intervals for a period of 10 minutes. Absorbance data was transformed to para-nitrophenolate concentration, accounting for the micro well volume light path length. Initial

velocities were calculated from linear least squares fits of the increase in sample para-nitrophenolate concentration with time data and reported in units of $\mu\text{M}\cdot\text{min}^{-1}$. Initial velocities were plotted against *p*NPP concentration for each of the rose bengal concentrations and the resulting dose response curves fitted globally to two noncompetitive inhibition models (equations 6 and 7, Supplementary Fig. S14; Supplementary Table S2).

Binding of Rose Bengal to DUSP5 PD (K_d) Determined by UV-Vis Spectroscopy

Binding of rose bengal to DUSP5 PD protein was measured directly by titrating samples containing rose bengal with DUSP5 PD protein (Fig. 9). In three separate experiments, the total concentration of rose bengal was kept constant at 1.08, 3.25 or 6.5 μM and titrated with increasing DUSP5 PD protein concentrations. The titrated protein was added in 5 μL volumes into quartz cuvettes containing 2 mL of the rose bengal in sample buffer containing 0.1 M Tris, 0.1 M NaCl pH 7.5 without or with 1 mM DTT. Final sample protein concentrations were 0, 1.0, 2.0, and 3.0 μM for 1.08 μM rose bengal samples, and 0, 0.5, 1.0, 1.5, 2.0, and 2.5 μM for samples that contained either 3.25 μM or 6.5 μM rose bengal. Absorbance was recorded over a wavelength range of 450 nm to 650 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific), with protein concentrations of interest without rose bengal as blanks. Upon addition of protein to the rose bengal, the absorption spectrum was red shifted in relation to the original sample containing rose bengal only. Difference spectra at each protein concentration were generated by subtracting the rose bengal samples without protein from samples containing both rose bengal and the DUSP5 PD protein. The absorbance difference values at 571 nm – 548 nm were generated from the difference spectra and plotted against the total protein concentration in the rose bengal sample. The resulting data was fitted to equation 8;

$$y = \frac{Abs_{max} * X}{W + X} \quad (\text{equation 8})$$

with y as the measured absorbance differences, Abs_{max} the calculated maximum absorbance difference value at equilibrium, X the total protein concentration, and W the protein concentration at which the absorbance value was one-half the maximum value. The data were fitted to obtain the Abs_{max} value, the absorbance value of fully bound rose bengal. Fractional absorbance values from each titration were determined by dividing the absorbance difference values (y) by the maximum absorbance difference value (Abs_{max}). The fractional values were then plotted versus the total protein concentrations. Since a considerable fraction of the protein was bound to the rose bengal, a quadratic equation was used to fit the binding data, which yielded an estimated dissociation constant K_d .

$$y = ((C_P + C_L + K_d) - \sqrt{(C_P + C_L + K_d)^2 - (4C_P C_L)}) / 2 \quad (\text{equation 9})$$

where y is the fraction of rose bengal bound to the protein, C_P is the total concentration of DUSP5 PD protein, which in our experiments was varied, C_L is the total concentration of rose bengal, which was kept constant during each experiment. Curves were fitted using GraphPad Prism v.6 (Fig. 9; Supplementary Table S3).

Spectral Analysis of Other Xanthene Dyes Binding to DUSP5 PD Protein

Interactions of DUSP5 PD protein with merbromin, erythrosin B, eosin Y, 2',7'-dibromofluorescein, 2',7'-dichlorofluorescein and fluorescein were studied spectrophotometrically (Supplementary Figs. S16-S18). Absorbance measurements of 4 μ M concentrations of each compound in assay buffer without and with 1 mM DTT were collected at 1 nm intervals between 400 and 600 using a Genesys 10S IV-Vis spectrophotometer (Thermo Scientific). Samples volumes were 2 mL in quartz cuvettes. Following the initial scan, DUSP5 PD protein was added to each compound sample to a final concentration of 4 μ M, and a second scan performed. Blank samples included assay buffer without and with DTT as well as assay buffer without and with DTT, containing 4 μ M DUSP5 PD protein. Spectral changes resulting from protein addition were examined by plotting the difference spectra of the without and with protein samples.

Nephelometry

Nephelometry was performed as described previously¹⁶, to determine the relative propensity of selected inhibitor compounds to aggregate in solution, based on the light scattering properties of the molecular aggregates (Supplementary Fig. S19). Such aggregation effects, which are common for dyes, are known to lead to the artefactual appearance of enzyme inhibition. Merbromin and eosin Y were tested for aggregation in a 96-well plate format, using substrate-free activity assay buffer without and with 1 mM DTT, at pH 7.5. Compound concentrations ranging from 1–100 μ M merbromin and 1–300 μ M eosin Y were generated by the addition of 4 μ L volumes of serially diluted compound solutions to 196 μ L assay buffer. Four to eight wells were used for each concentration and data were collected using a BMG NEPHELOStar Plus, equipped with a 635 nm laser

Docking and DFT Calculations

The atomic coordinates of DUSP5 were obtained from the Protein Data Bank (PDB ID: 2G6Z). Structures of the ligands were generated using OpenBabel software¹⁸ from SDF files obtained from PubChem and optimized using density functional theory (DFT) calculations at the B3LYP/def2-SV(P) level of theory (Fig. 3). Both protein and ligands were preprocessed for docking using the AutoDock tools¹⁹. The protein structure was prepared by deleting chains B and C and sulfate anions from the protein structure, adding of hydrogens, assigning partial Gasteiger charges and merging nonpolar hydrogens and their charges with the parent carbon atom. The structures of ligands were preprocessed by assignment of Gasteiger atomic charges and torsions, merging nonpolar hydrogens and their charges with the parent carbon atom.

The AutoDock program¹⁹ was applied for the ligand docking to the active site of the DUSP5. The docking grid dimensions were 20 Å $\times$ 20 Å $\times$ 15 Å with the point separation of 0.2 Å. The Lamarckian Genetic Algorithm was employed for docking ($N = 50$).

Electrostatic potential surfaces of DUSP5 (shown in Fig. 10) were calculated and visualized using Chimera with the APBS plugin²⁰. A series of the B3LYP/def2-SV(P) single-point calculations were performed at the optimized geometries of the ligands to compute atomic charges fit to the electrostatic potential at points selected according to the CHelpG

algorithm. Electrostatic potential surfaces (from -0.25 to -0.15 e⁻) as well as the highest occupied molecular orbitals (HOMOs), lowest unoccupied molecular orbitals (LUMOs) of ligands (Table 3) were mapped using Terse²¹. All DFT calculations were done with the Gaussian 16 package of programs²².

RESULTS

Identification of Merbromin in the DUSP5 Screen of FDA Drugs and Analogs

Previously, we had developed and validated a *para*-nitrophenol phosphate (*p*NPP) substrate-based high throughput assay for DUSP5, with a Z' factor of 0.73¹⁶. Using this assay, we screened the Prestwick library of 1,280 compounds, which included FDA-approved drugs, to identify compounds that might block or enhance DUSP5 activity, using the DUSP5-phosphatase domain (DUSP5 PD). Of the 1,280 compounds screened (Supplementary Fig. S1a), we identified 9 compounds (Levodopa, Cyanocobalamin, Primaquine diphosphate, Propidium iodide, Merbromin, Tetrahydroxy 1,4-quinone monohydrate, Lymecycline, Eseroline fumarate salt, and Anthracin) that showed inhibitory activity with DUSP5. Of the 9 compounds, Lymecycline was not commercially available, and therefore we tested only 8 compounds for activity in the secondary assay with the DUSP5-relevant *p*ERK substrate (Supplementary Fig. S1b). We performed a non-cell based western blot analysis where we incubated pure DUSP5 protein and *p*ERK in the presence or absence of the putative inhibitor, and compounds identified in the *p*NPP screen were tested for inhibition *p*ERK dephosphorylation (Supplementary Fig. S1c). The most potent inhibitor was merbromin, which is also known as mercurochrome (a topical antiseptic). For this reason, merbromin – and its known xanthene dye analogs – became the focus of our studies moving forward.

Because merbromin has a mercury atom in its structure, which is a safety concern for a drug lead, we purchased or synthesized several analogs of merbromin which lack the mercury element, and which also have different numbers and types of halogen substitutions. These include eosin Y, which is identical to merbromin but lacks the mercury atom, as well as 2', 7'-dibromofluorescein, 2', 7'-dichlorofluorescein, erythrosinB and fluorescein (Fig. 1). Dyes such as eosin Y have been previously used for treatment of vascular anomalies¹⁴, and in other prior studies^{23,24}. The chemical structure of merbromin and these various merbromin analogs, bromo-, chloro- or iodo-halogenated xanthenes, are shown in Figs. 1a and b. Compound dose response experiments were performed with merbromin, eosin Y, 2', 7'-dibromofluorescein, 2', 7'-dichlorofluorescein, erythrosine B, rose bengal and fluorescein. Fig. 2a shows DUSP5 PD initial velocity inhibition curves fitted (equation 2) for estimation of IC₅₀ for merbromin. Inhibition profiles were generated with no incubation period (initial assay) and following a 30 and 60 mins incubation of merbromin with DUSP5 PD enzyme in the dark at room temperature. A decrease in enzyme activity was observed with increasing merbromin dose; and, potency increased with incubation time, indicated as a leftward shift in the IC₅₀ curve. When DTT was included in the assay buffer (Fig. 2b), merbromin became less potent and the leftward shift in the IC₅₀ curve with time was blunted. A similar result was observed with eosin Y (Fig. 3a and b), although it was less potent than merbromin. 2', 7'-dibromofluorescein (Supplementary Fig. S2a), inhibited DUSP5 PD in a dose-dependent manner, displayed increased potency with time of exposure, and exhibited decreased potency

in the presence of DTT. 2',7'-dichlorofluorescein (Supplementary Fig. S2b.), erythrosin B (Supplementary Fig. S2c), and rose bengal (Supplementary Fig. S2d) also inhibited DUSP5 PD activity in a dose-dependent manner; however, for these three xanthenes, the time-dependence for inhibition was only modest and there was almost no effect of DTT. Fluorescein did not inhibit DUSP5 PD activity at concentrations up to 300 μM (Supplementary Fig. S3). Table 1 lists the estimated IC_{50} values generated from the dose response curves fitted to equation 2, without and with 1 mM DTT in the assay buffer and following preincubations with inhibitor of 0, 30 and 60 min. Rose bengal showed the greatest potency of all compounds tested.

Dose response studies were also performed with merbromin and eosin Y using full length DUSP5 and *p*ERK as the substrate. Figure 4a shows a representative immunoblot of *p*ERK and total ERK following reaction of DUSP5 with *p*ERK and over a range of merbromin concentrations (0.1 μM to 5 mM) in the reaction mixture. Immunoblot densitometry analysis of these gels resulted in dose response curves that were fitted (equation 1) in Figs. 4b and c, yielding IC_{50} estimations of $0.6 \pm 0.4 \mu\text{M}$ for merbromin and $12.7 \pm 3.0 \mu\text{M}$ for eosin Y.

Determination of Time-dependent Kinetics through Reaction Progress Curve Analysis

Due to the time-dependent inhibition observed for several of the study compounds, additional studies were performed to help elucidate the mechanism(s) of compound inhibition. DUSP5 PD dose response curves with 0 to 50 μM merbromin without (Fig. 5a) and with 1 mM DTT (Fig. 5b) in the assay buffer were generated using *p*NPP as the substrate. Reaction progress curves were recorded for 120 min and non-linear fits of each curve were performed using equation 3, a model for irreversible enzyme inactivation represented as line fits to the data. k_{obs} , the pseudo first-order rate constants, were computed from the fits of the *para*-nitrophenolate concentration versus time data. The relationship of k_{obs} to inhibitor concentration helped define the inhibition mechanism. Replots of the k_{obs} versus merbromin concentration data (Fig. 5c) revealed that without DTT, k_{obs} was a hyperbolic function of merbromin concentration, suggesting a two-step inhibition mechanism (Scheme 1, mechanism 2). The hyperbolic data (Fig. 5c) were fitted to equation 4 (solid line), which yielded kinetic inhibition constant values for k_{inact} (inactivation rate constant), the rate constant for the conversion of the initial complex to the final covalent complex with a model fit value of $1.4 \times 10^{-3} \text{ s}^{-1}$, and K_I , the equilibrium dissociation constant for the first step of reversible non-covalent binding of the compound to the enzyme, to form the initial non-covalent complex. The overall strength of the binding affinity is usually measured by the inhibition constant K_I , which could be thought of as the equilibrium dissociation constant of this initial complex; and, it has a value of $3.3 \times 10^{-5} \text{ M}$, computed from the model fit of the hyperbolic data in Figure 5c. Evaluation of irreversible enzyme inhibitors often relies on the ratio k_{inact}/K_I ($42.4 \text{ M}^{-1}\text{s}^{-1}$) as a measure of compound potency for two step irreversible enzyme inactivation. It is a second order rate constant for irreversible inhibition, so larger numbers indicate more effective inhibition. The merbromin with DTT plot of k_{obs} as a function of merbromin concentration was linear (Fig. 5c, dashed line), with an intersection at the origin. The fit suggests a single-step inhibition mechanism (Scheme 1, mechanism 1). The slope from the data fit (6.0 M^{-1}) is descriptive of k_{inact}/K_I and a measure of compound potency for single step irreversible enzyme inactivation.

Progress curves over a range of eosin Y concentrations from 0 to 100 μM without and with DTT (Fig. 6a and 6b) were fitted in a similar manner as merbromin, resulting in hyperbolic and linear relationships of k_{obs} with eosin Y concentration varied without and with DTT present, respectively (Fig. 6c). Calculated k_{inact} , K_I and k_{inact}/K_I values without DTT present were $2.0 \times 10^{-4} \text{ s}^{-1}$, $4.3 \times 10^{-5} \text{ M}$ and $6.6 \text{ M}^{-1}\text{s}^{-1}$, respectively, and with DTT present k_{inact}/K_I was $1.0 \text{ M}^{-1}\text{s}^{-1}$. Supplementary Figs. S4-S10 show complete 120 min reaction progress curve data sets for each of the tested compounds. Merbromin (Supplementary Fig. S4), eosin Y (Supplementary Fig. S5), 2',7'-dibromofluorescein (Supplementary Fig. S6), 2',7'-dichlorofluorescein (Supplementary Fig. S7) and erythrosin B (Supplementary Fig. S8) curves were fitted to equation 3, indicated as lines through the data, to determine k_{obs} . Panels in (a) represent experiments performed without DTT, and panels in (b) represent experiments performed with 1 mM DTT in the assay buffer. The model-generated k_{obs} values were then replotted as a function of inhibitor concentration (panels in c), and fitted with equation 4 if the relationship was hyperbolic, or a linear least-squares model for linear relationships, to generate k_{inact} , K_I and k_{inact}/K_I values. Rose bengal (Supplementary Fig. S9) and fluorescein (Supplementary Fig. S10) reaction progress curves were not fitted due to the linearity of the reaction at the concentrations tested. Kinetic model fit parameters for the irreversible DUSP5 PD inactivation and inactivation mechanisms for the datasets and fittings are summarized in Table 2. The model fit indicated that merbromin and eosin Y, in the absence of DTT, inhibited DUSP5 PD by a two-step inhibition mechanism (Scheme 1), with binding and dissociation of the xanthene inhibitor occurring before the formation of an inactive enzyme-compound complex. All other compounds, as well as merbromin and eosin Y with DTT, inactivated DUSP5 PD in a single step mechanism (Scheme 1), with inactivation directly proportional to the concentration of the compound. The most effective time-dependent inhibitor was merbromin, with a k_{inact}/K_I value of $47.08 \pm 4.66 \text{ M}^{-1}\text{s}^{-1}$; although, this rate decreased 7.6-fold in the presence of DTT. In the absence of DTT, eosin Y and erythrosin B were also good inhibitors, but roughly 10-fold less effective than merbromin. Rose bengal is not a time-dependent irreversible inhibitor, so is not being compared to these other xanthene compounds in Table 2 (i.e. it has no value for k_{inact}/K_I).

Steady-state Inhibition Mechanisms

The observation that DUSP5 PD inhibition by erythrosin B had little time dependence without or with DTT led us to examine additional and more traditional steady state inhibition mechanisms. Initial velocity inhibition profiles of erythrosin B were obtained by measuring initial velocities at varied concentrations of substrate ($p\text{NPP}$) and inhibitor (erythrosin B) in assay buffer containing DTT. The inhibition profile was fitted globally to the equation for competitive inhibition (equation 5) using GraphPad Prism 6. Figure 7a shows DUSP5 PD initial velocities plotted as a function of $p\text{NPP}$ concentration (symbols) over a range of erythrosin B concentrations, with the globally fitted model data displayed as solid lines. Parameter estimates are presented in the Fig. 7 legend. The estimated K_I was $10.87 \mu\text{M}$ (Fig. 7), compared with an IC_{50} value of $19.1 \mu\text{M}$ (Supplementary Fig. S2c and Table 1). Figure 7b shows a double reciprocal plot with each erythrosin B concentration sharing a common V_{max} value, consistent with a competitive inhibition mechanism where erythrosin B binds in the $p\text{NPP}$ active site pocket (Fig. 10a).

Rose Bengal, another study compound that displayed no time-dependent inhibition or DTT sensitivity has been reported previously as a photo-reactive compound^{25,26}. Fig. 8a shows DUSP5 PD initial velocity data plotted as a function of *p*NPP concentration (symbols) over a range of rose bengal concentrations (0, 1, 2.5 and 3.5 μ M), and that had been incubated in the dark for 15 mins before substrate addition. The inhibition profile was fitted globally to a noncompetitive inhibition model with a single K_i (equation 6) and represented as solid lines, yielding best fit kinetic estimates for V_{max} , K_m and K_i (Supplementary Table S1). Fig. 8b shows initial velocity profile data following rose bengal incubation over the same concentration range as above with DUSP5 PD exposed to a light source for 15 mins before the addition of substrate. The inhibition profile was fitted globally to a noncompetitive inhibition model with a single K_i as above. The rose bengal K_i value of $2.27 \pm 0.14 \mu$ M for the dark reaction decreased 4.5-fold to $0.54 \pm 0.03 \mu$ M when the inhibition reaction was carried out after a pre-incubation with light. Figs. 8c and 8d are the same velocity profiles as Figs. 8a and 8b, respectively, but each fitted globally to a noncompetitive inhibition model having two K_i values (equation 7): K_{is} , the slope inhibition constant, is the dissociation constant of rose bengal with respect to the free enzyme and K_{ii} , the intercept inhibition constant, is the rose bengal dissociation constant with respect to the enzyme-substrate complex. Model parameter estimates of V_{max} , K_m , K_{is} and K_{ii} are also presented in Supplementary Table S1. Supplementary Fig. S11 shows six independent velocity studies with rose bengal as inhibitor, and as described for Figs. 8a and 8b. Experiments in panels for Supplementary Figs. S11a, S11b, and S11c were performed following 15 min dark incubations of rose bengal with DUSP5 PD before substrate addition while experiments in Supplementary Figs. S11d, S11e, and S11f were performed following 15 min exposure of the rose bengal with DUSP5 PD to a light source. All data were fitted to equation 6, and the resulting kinetic parameters reported in Supplementary Table S1. Supplementary Fig. S12, panels a, b, c, d, e, and f contain the same data as the respective Supplementary Fig. S11 panels, but were fitted using equation 7, with kinetic parameters reported in Supplementary Table S1. Light exposure decreased DUSP5 PD activity at all rose bengal concentrations tested. Dark and light exposed sample data modeled with equation 6 showed significantly different K_i values ($2.27 \pm 0.14 \mu$ M vs $0.54 \pm 0.03 \mu$ M, mean $\pm$ S.D.) for dark and light exposures, respectively, while data modeled using equation 7 showed significant differences between K_{is} ($0.35 \pm 0.04 \mu$ M vs. $0.08 \pm 0.00 \mu$ M) and K_{ii} ($2.25 \pm 0.10 \mu$ M vs. $0.55 \pm 0.02 \mu$ M, mean $\pm$ S.D.) values for dark and light exposed samples, respectively. In contrast to the noncompetitive inhibition by rose bengal (Fig. 8), erythrosin B in Fig. 7b shows a double reciprocal plot with each erythrosin B line sharing a common V_{max} value, suggesting a competitive inhibition mechanism for erythrosin B.

Temperature Effects from Illumination—The broad emission spectrum of the incandescent lamp is potentially problematic since high wavelength emissions can result in sample heating and impact experimental and treatment results. The heat emitted from the incandescent light source and the possible effect on protein activity was the motivation for performing the sample illuminations in a chromatography refrigerator. The effect of incandescent light on sample temperature is shown in Fig. S13. The ambient temperature inside the refrigerator was well-maintained when the light was off, ranging from $3.9 \pm 0.1 \text{ }^\circ\text{C}$ to $5.1 \pm 0.1 \text{ }^\circ\text{C}$ over the recording period. When the light was turned on, ambient

temperature cycled between 5.6 ± 0.1 and 7.1 °C. Assay sample temperatures exposed to light without and with 3.5 μM rose bengal steadily increased with time from 5.5 ± 0.2 to 9.2 ± 0.1 °C, and 5.2 ± 0.5 to 9.6 ± 0.4 °C, respectively. Complete shielding of samples with aluminum foil somewhat slowed the increase in temperature. In any case, sample temperatures did not exceed 10°C throughout the 15 minute illumination period.

LED Illumination Kinetic Studies—In order to minimize sample heating (and avoid the need for sample cooling) during the illumination period, similar experiments were performed using a lower intensity LED light source. Illumination experiments were performed for 30 minutes at room temperature with a calculated light density value of 0.3 mW/cm^2 and a measured illuminance value of 825 lux, with no increase in sample temperature observed over the 30 minute LED light exposure period. Figure S14 shows DUSP5 PD initial velocity plotted as a function of $p\text{NPP}$ concentration over a range of rose bengal concentrations for samples that had been incubated in the dark, Fig. S14a and S14c, or illuminated for 30 minutes with the LED, Fig. S14b and S14d. Solid lines are global fits to noncompetitive inhibition models with a single or dual K_i s (equations 6 and 7). Kinetic data are reported in Table S2. Enzyme velocities of the LED-illuminated samples containing rose bengal were inhibited to a greater degree than the samples that were shielded from light. While V_{max} and K_m values are similar between the dark and light incubation conditions, the K_i values for the illuminated samples are about half that of samples that were incubated in the dark.

Headspace Gas Sparging Effects on Kinetics—Because singlet oxygen production has been implicated in rose bengal photosensitization, we investigated the impact of oxygen on light-dependent DUSP5 PD inhibition in the presence of rose bengal. Figure S15a shows DUSP5 PD velocities for samples containing 1 μM enzyme and exposed to room air, or 1 μM enzyme and 1 μM rose bengal that were gassed with compressed air, nitrogen, or oxygen prior to light exposure. Enzyme velocities were significantly decreased in all illuminated samples containing rose bengal when compared with the illuminated sample without rose bengal. Samples gassed with nitrogen had significantly higher velocities than samples gassed with either compressed air or oxygen. Samples gassed with oxygen had significantly lower activities than each of the other conditions. Gassing samples containing enzyme but no rose bengal prior to light exposure had no effect on enzyme activity (Fig. S15b).

Electronic Interaction of Rose Bengal with DUSP5 PD

Figures 9a and 9d show the visible absorption spectra of 6.5 μM rose bengal in assay buffer without and with 1 mM DTT, respectively, with DUSP5 PD protein titrated into each sample to final concentrations of 0.5 , 1.0 , 1.5 , 2.0 and 2.5 μM . Addition of DUSP5 PD protein resulted in a red shift in the spectrum. The spectral shift was used to characterize the electronic interactions between rose bengal and DUSP5 PD, and to quantify binding affinity directly. Difference spectra (Fig. 9b and 9e) were generated by subtracting the rose bengal sample spectrum without protein from the spectra following protein addition, resulting in positive peak values at 571 nm and negative peak values at 548 nm. This red shift of 23 nm is due to the change in electronic state of rose bengal induced by protein interactions. The differences between these absorbance values were normalized using equation 8 to obtain the

fraction of rose bengal that was bound to the DUSP5 PD at the respective concentrations of added DUSP5 PD protein. The fraction of bound rose bengal was plotted against DUSP5 PD concentration (Fig. 9c and 9f) and fitted to the quadratic equation for binding (equation 9), indicated as the solid and dashed lines. The quadratic equation generates a model solution for K_d , the dissociation constant for rose bengal with respect to DUSP5 PD. Additional experiments were performed in a similar manner using 1.08 μM rose bengal and without and with DTT present and 0, 1.0, 2.0 and 3.0 μM DUSP5 PD (Supplementary Fig. S16), and 3.25 μM rose bengal without and with 1 mM DTT and 0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM DUSP5 PD protein (Supplementary Fig. S17). Spectral shifts were analyzed using equation 9 as described above and the resulting K_d values for rose bengal binding to DUSP5 PD reported in Supplementary Table S3. There was no significant difference between the three trials with and without DTT in the assay buffer, with average values of $0.69 \pm 0.17 \mu\text{M}$ vs. $0.81 \pm 0.56 \mu\text{M}$, respectively. Within error limits, DTT had no effect on rose bengal binding affinity, and the K_d values determined from the spectral shift assays were close to the value of 0.54 μM obtained with steady state kinetics (Fig. 8).

Spectral shifts were also used to study merbromin, erythrosin B, eosin Y, 2',7'-dibromofluorescein, 2',7'-dichlorofluorescein, and fluorescein binding to DUSP5 PD protein without and with 1 mM DTT in the assay buffer (Supplementary Figs. 18a, b, c, d, e, and f, respectively). Each compound was prepared to a concentration of 4 μM and absorbance recorded between 400 and 600 nm. Absorbance was recorded again following the addition of 4 μM DUSP5 PD protein. Spectra with protein absent were subtracted from spectra with protein, resulting in the difference spectra shown in Supplementary Fig. S18. The largest spectral shift was observed upon addition of protein to the merbromin sample without DTT. Addition of DTT blunted the merbromin spectral shift (Supplementary Fig. 18a), suggesting a lower binding affinity when DTT was present. Erythrosin B and eosin Y (Supplementary Figs. 18b and c) were both spectrally shifted upon protein addition, however the shifts were not diminished in the presence of DTT. The 2',7'-dibromofluorescein spectra shifted slightly upon protein addition both with and without DTT present (Supplementary Fig. 18d), while 2',7'-dichlorofluorescein and fluorescein showed no spectral differences under the test conditions.

Merbromin and Eosin Y Nephelometry

Nephelometry is a technique for measuring relative particle aggregation in solution based on the light scattering of molecular aggregates. Compound aggregates could potentially inhibit enzyme activity through nonspecific mechanisms. We tested merbromin and eosin Y in assay buffer without and with 1 mM DTT to investigate whether the blunting of DUSP5 PD inhibition in the presence of DTT resulted from aggregation effects. Supplementary Fig. S19 shows aggregation, measured in relative nephelometric units, plotted against the log concentration of merbromin (Supplementary Fig. S19 a,b) and eosin Y (Supplementary Fig. S19 c,d) with progesterone as the control. The point of inflection reflects the concentration of compound at which aggregation is initiated. The addition of DTT had little effect on merbromin and eosin Y aggregation, suggesting that the difference in DUSP5 PD activity in the presence of these compounds without and with DTT in the assay buffer is not due to a difference in aggregation.

Computational Modeling of the Halogenated Xanthene Dyes

To provide a better understanding of the structural and electronic basis of inhibition of DUSP5 inhibition by the xanthene dyes, docking and electronic calculations were performed. Docking studies for the xanthene dyes in Fig. 1, using AutoDock, showed little correlation between calculated binding energy and IC₅₀ values (Supplementary Fig. 20a). This is likely because of the covalent or irreversible ligand-protein binding (Figs. 5, 6; Scheme 1) and electronic effects (see Fig. 9) which are important for binding the xanthene dyes to proteins, but are not dealt with adequately by force fields that are based on classical molecular mechanics. For this reason, DFT calculations were used to determine electrostatic potentials, as well as LUMO and HOMO shapes and energies (summarized in Table 3). Interestingly, there is a good correlation between IC₅₀ values and LUMO energy (Supplementary Fig. 20c), with lower reduction potentials correlated with lower IC₅₀ values. This correlation would be consistent with the known propensity of halogenated xanthene dyes like rose bengal to form radical anions, by oxidizing thiols (to the thiyl radical, RS·)²⁷ or other protein residues, like arginines²⁸, upon light exposure.

While docking energies based on classical mechanics will not be entirely accurate if electronic interactions, like those described above, are important contributors to binding energy, it is interesting that the halogenated xanthenes docked in to the DUSP5 active site in different orientations. For example, merbromin docked in an orientation that spanned both the active site and the secondary site pockets (Fig. 10b), whereas rose bengal docked in an orientation that occupied only the secondary pocket, with its xanthene ring (possibly as a radical anion, charge transfer complex). That latter binding mode is consistent with rose bengal behaving as a noncompetitive inhibitor (i.e. it is possible for *p*NPP to be bound, simultaneously, to the active site pocket while rose bengal is in the secondary pocket).

DISCUSSION

In this study, we have combined our structure, mechanism and molecular modeling knowledge from previous studies of DUSP5 and applied it to the identification of FDA-approved (and related) compounds that can be repurposed for treatment of vascular anomalies. The salient features and key results include: (a) the identification of three drug lead compounds, merbromin, eosin Y and rose bengal that target DUSP5, (b) the slow-binding irreversible mechanism of inhibition, and (c) the role of photoactivation in facilitating enzyme inhibition. Rose bengal, eosin Y, merbromin and other xanthenes typically interact with DUSP5 by binding via a charge-transfer complex to one or both phosphate binding pockets (Fig. 10), which contain positively charged residues; and, enzyme inactivation is in some cases irreversible, likely due to a light-induced oxidation of the enzyme by the xanthene dye.

Our previous work on DUSP5 inhibitors pointed to the key feature of anionic groups on a lead inhibitor that bound in the active site near the catalytic Cys263 (mutated to serine in the structure), and in a secondary site 7.2 Å away from the active site¹⁶. Both of these binding pockets are positively charged and accommodate ligands with negative charges. Initially, sulfates had been proposed to occupy the binding pockets that are presumably occupied by the phosphate groups on the substrate, pERK¹⁴. For the ERK2 substrate, the pThr-Glu-pTyr

tripeptide region of the ERK2 activation loop presumably occupies this region in the DUSP5 PD⁷. In general, inhibitors are expected to occupy one or both of these basic binding pockets, which are located 7.2 Å apart (Fig. 10a).

In the FDA-approved compound screen, we discovered merbromin and subsequently several analogs of merbromin, including eosin Y and rose bengal, which became the focus of this study (Fig. 1). All of these DUSP5 inhibitors possess some negative charge, with a negative electrostatic potential (Table 3); and, some can form radical anions due to their very low LUMO energies (Table 3). Accordingly, they might be expected to have affinity for the positively charged binding pockets of DUSP5 (Fig. 10). Merbromin is more potent than eosin Y in inhibition assays using the phosphatase domain of DUSP5, and the *p*NPP substrate (4.1 μM vs. 37.6 μM, Table 1). Merbromin and eosin Y are even more potent inhibitors in the more biologically-relevant assay with full length DUSP5 using *p*ERK as substrate, with IC₅₀ values of 0.6 μM and 12.7 μM, respectively (Fig. 4). Merbromin and eosin Y have similar chemical structures – differing only in that merbromin has a mercury atom present, whereas eosin Y does not. The added potency of merbromin is likely due to the presence of the mercury Lewis acid properties. Even though merbromin is more potent, eosin Y is favored as a drug lead since it lacks the mercury atom. Merbromin raised toxicity concern due to the presence of the mercury atom, and the FDA has since banned the sale of Mercurochrome in the U.S. Based on these observations, a structure-activity relationship study was undertaken by varying halogen substitutions, with the series of halogenated xanthenes shown in Fig. 1. Interestingly, some of these compounds showed time-dependent inhibition (Table 1; Figs. 2, 3) and a sensitivity to DTT. If enough time is elapsed to observe full inhibition, the relative inhibitor potency based on IC₅₀ is: rose bengal (2.3 μM) > merbromin (4.1 μM) > erythrosin B (14 μM) > eosin Y (37.6 μM). For the time-dependent rate of inhibition, merbromin was most effective with a k_{inact}/K_I value of 47 M⁻¹s⁻¹, followed by erythrosin B (4.65 M⁻¹s⁻¹) and eosin Y (6.06 M⁻¹s⁻¹) (Table 2, Figs. 5, 6). Although, this inhibition is decreased in the presence of DTT for merbromin and eosin Y. The reason for this DTT effect is not clear, although it likely is caused by a reaction with the xanthene dyes, since it is known that xanthenes react with and can oxidize thiol groups to thiyl radicals²⁷. Indeed, such redox and electronic properties likely play a critical role in the mechanism of inhibition of DUSP5 by the xanthene dyes.

The xanthenes shown in Fig. 1 differ in the nature and degree of halogenation, which will have a significant effect on their electronic properties. Those effects, in terms of electrostatic potential and LUMO and HOMO energies are summarized in Table 3. Interestingly, there is a good correlation between LUMO energy and inhibitor potency, with compounds having a lower LUMO energy also having a lower IC₅₀ (Supplementary Fig. S20c). A lower LUMO energy means the compound could also be more easily reduced to the radical anion, which is known to occur with xanthene dyes^{29,30}. This may explain why rose bengal, which has the most electropositive charge density, is able to bind in a very positive protein pocket – if in fact it must first be reduced to the radical anion. Indeed, xanthenes like eosin Y and rose bengal have been reported to act as strong electron acceptors when they are in a photoexcited state, oxidizing thiols to thiyl radicals²⁷, and oxidizing lysine and arginine residues²⁸. Consistent with this mechanism for DUSP5 inhibition, we have shown that rose bengal is a more potent inhibitor if it is first pre-treated with light (2.27 μM (dark) vs. 0.54

μM (light), Fig. 8). Furthermore, binding of rose bengal by DUSP5 protein also causes a spectral shift (Fig. 9), consistent with formation of a charge transfer complex (with the radical anion of rose bengal). Similar red shift spectral changes have been reported previously, for rose bengal binding to proteins or in forming model donor-acceptor complexes^{28,31}. Fitting of the spectral titration data for rose bengal gave a K_d of $0.69 \pm 0.17 \mu\text{M}$ (Fig. 9), similar to the K_i obtained from the steady-state enzyme kinetic inhibition study ($0.54 \mu\text{M}$).

Docking studies suggest that the xanthene compounds can bind in the active site, the secondary site, or both pockets (Fig. 10). Merbromin, erythrosin B and eosin Y are predicted to bind and block both pockets, whereas rose bengal is predicted to bind only in the positively charged secondary site. Consistent with these docking predictions, steady state enzyme kinetic inhibition studies (Figs. 7, 8; Supplementary Figs. S11 and S12; Supplementary Table S1) indicate that while erythrosin B is a competitive inhibitor (i.e. inhibits by blocking *p*NPP binding in the active site), rose bengal is a noncompetitive inhibitor (i.e. does not inhibit exclusively by blocking *p*NPP binding in the active site).

The light activation of rose bengal inhibition is likely due to its known propensity to become a strong oxidizing agent in its photoactivated state, able to then form a radical anion (and consequently oxidizing the protein, irreversibly^{27,28}). Accordingly, light-activation makes rose bengal a more potent inhibitor, and (because noncompetitive inhibition is observed) it has high affinity binding for binding both the substrate free and substrate bound forms of DUSP5 (i.e. rose bengal does not fully block the active site, as in Fig. 10c). In summary, rose bengal behaves differently than erythrosin B and other xanthenes in that it can also inhibit under saturating substrate (V_{max}) conditions; so, it can bind to the preformed DUSP5-*p*NPP Michaelis complex, and still cause inhibition. This would be consistent with it being able to bind in the secondary pocket, and not block the active site pocket directly, as the docking calculation suggest (Figs. 10a and 10c). Its ability to inhibit the enzymatic reaction, when not physically blocking the active site pocket, could be due to irreversible enzyme oxidation (e.g. electron transfer from the active site cysteine thiol to the rose bengal bound in the secondary site, Fig. 10).

Both rose bengal and eosin Y could irreversibly inhibit the DUSP5 enzyme if they were to oxidize it (e.g. cysteine or arginine), while forming the radical anion on the xanthene ring. Or, if the radical anion could form before binding to DUSP5 (our preliminary studies show reaction with oxygen increase inhibition, Supplementary Fig. S15), then that rose bengal could bind and inhibit reversibly. We suspect the one- or two-step slow binding inhibition that was observed (Scheme 1), and the DTT suppression of inhibition, are due to effects on the formation of the radical anion. It is possible that rose bengal could inhibit DUSP5 by multiple mechanisms; although, it is clear that both light and oxygen increase inhibitor potency. Irrespective of the actual mechanism of DUSP5 inhibition, the ability of rose bengal – upon exposure to light – to form a reactive radical anion has led to its use in photodynamic therapies^{29,32}. A similar photodynamic therapy approach could be taken for treating vascular diseases like hemangiomas, by targeting DUSP5 with topically-applied rose bengal, or other light-activated xanthene dyes.

There is precedent for clinical applications of xanthene dyes. While clinical applications of rose bengal have focused on cancer ²⁹, eosin Y has shown efficacy in the clinic for hemangiomas ¹⁴; and, our studies suggest that one of eosin Y's targets in hemangioma could be DUSP5. An additive such as eosin Y would be inexpensive to make, and provide a novel alternative to patients suffering from vascular anomalies, and already has toxicology and safety study data available from the FDA since it is approved as D&C Red No. 23, with an LD₅₀ > 10 mmol/kg ³⁴.

In terms of potential clinical relevance of our results, these xanthene dye compounds will be optimized further in future structure-based drug design studies, and mechanistic studies will continue as well. But, eosin Y could be efficacious in disease settings in the shorter term, by repurposing, given its current clinical use. Likewise, rose bengal (referred to commercially as "PV-10") is currently in clinical trials for treating liver cancer (NCT00986661) and metastatic melanoma (NCT00521053), and could also be repurposed for additional clinical uses like treating vascular anomalies. Thus, the halogenated xanthenes have a unique mechanism of inhibition of DUSP5, and may potential clinical applications, including in photodynamic therapy for treating vascular anomalies on the surface of the skin. This may be a therapeutically viable option, given that vascular malformations are currently already being treated with laser therapy ³⁵ Future studies in our laboratory will be directed to further define the mechanism of DUSP5 inhibition, and to developing these xanthene dyes as photodynamic therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Xanthene-ring based merbromin and its analogs all inhibit DUSP5 *in vitro*, with relative potencies: rose bengal > merbromin > erythrosin B > eosin Y
- Inhibition is time-dependent, with enzyme inhibition increasing over time and fitting best to a slow-binding model of irreversible enzyme inactivation.
- Inhibition potency is correlated with the xanthene dye's LUMO energy, which affects ability to form light-activated radical anions, the likely active inhibitor form.
- Rose bengal inhibition is light-dependent, with a K_d of 690 nM.
- Xanthene dyes like rose Bengal could be used in photodynamic therapy, for treating vascular diseases that respond to DUSP5 inhibition.

Fig. 1. Chemical structures of merbromin and merbromin analog compounds.

(a) Chemical structures of merbromin and analog study compounds. (b) The respective merbromin analog compound substituent groups located at positions R1, R2, and R3 are presented in the table.

Fig. 2. Merbromin IC₅₀ determinations for DUSP5 PD.

The percentage of residual DUSP5 PD activity measured in assay buffer (a) without and (b) with 1 mM DTT, utilizing *p*NPP as the substrate in the presence of 0.3 – 50 μ M merbromin without pre-incubation (initial assay), and again following 30 and 60 minute incubations with merbromin. Dose response curves ($n = 3$ for each condition) were fitted to equation 2 which yielded IC₅₀ values (mean $\pm$ SD) in buffer without DTT of (a) $16.1 \pm 0.7 \mu\text{M}$, $6.5 \pm 0.2 \mu\text{M}$, and $4.1 \pm 0.1 \mu\text{M}$, and with DTT IC₅₀ values of (b) $30.9 \pm 0.9 \mu\text{M}$, $23.6 \pm 0.6 \mu\text{M}$, and $19.7 \pm 0.6 \mu\text{M}$, for the initial assay and the 30 and 60 min. incubations, respectively.

Fig. 3. Eosin Y IC₅₀ determinations for DUSP5 PD.

The percentage of residual DUSP5 PD activity measured in assay buffer (a) without and (b) with 1 mM DTT, utilizing *p*NPP as the substrate in the presence of 0.3 – 100 μ M eosin Y without pre-incubation (initial assay), and again following 30 and 60 minute incubations with eosin Y. Dose response curves ($n = 3$ for each condition) were fitted to equation 2 which yielded IC₅₀ values (mean $\pm$ SD) in buffer without DTT of (a) $> 100 \mu$ M, $63.3 \pm 6.9 \mu$ M, and $37.6 \pm 4.5 \mu$ M, for the initial assay and the 30 and 60 min. incubations, respectively. IC₅₀ values were $>100 \mu$ M for reactions with eosin Y when the assay buffer contained 1 mM DTT.

Fig. 4. Inhibition of GST-DUSP5-mediated pERK dephosphorylation by merbromin and eosin Y. Panel (a) shows Western blot images generated following the reaction of active ERK2 with GST-DUSP5 samples that were incubated with 1×10^{-7} to 5×10^{-3} M merbromin. In addition, active ERK samples without added GST-DUSP5, and samples with ERK and GST-DUSP5 but without merbromin were generated as controls. Total ERK is shown for comparison. Pooled densitometry analysis of three separate experiments with merbromin and eosin Y are shown in panels (b) and (c), respectively. Model estimate $IC_{50} \pm SE$ values for merbromin, eosin Y from global data analysis (equation 1) were $0.6 \pm 0.4 \mu\text{M}$ and $12.7 \pm 3.0 \mu\text{M}$, respectively.

Fig. 5. Kinetic evaluation of DUSP5 PD inhibition with merbromin.

DUSP5 PD activity, measured as the generation of *p*-nitrophenolate product from *p*NPP substrate in assay buffer (a) without DTT and (b) with 1 mM DTT, was monitored over a two hour period in the presence of 0 – 50 μM merbromin. Each condition was fitted to equation 3, represented as solid lines, from which the apparent first order rate constants for inhibition k_{obs} were obtained at each merbromin concentration, (c) k_{obs} values generated from the –DTT and +DTT experiments were replotted as a function of inhibitor concentration. The hyperbolic line, descriptive of a two-step irreversible inactivation mechanism of DUSP5 PD by merbromin (Scheme 1, mechanism 2) in the absence of DTT, represents a nonlinear least-squares fit to equation 4, yielding values for k_{inact} , K_I and k_{inact}/K_I equal to $1.4 \times 10^{-3} \text{ s}^{-1}$, $3.3 \times 10^{-5} \text{ M}$, and $42.4 \text{ M}^{-1}\text{s}^{-1}$, respectively. The straight line through the +DTT data constrained through the origin represents a linear least-squares fit describing a single-step mechanism for the irreversible inactivation of DUSP5 PD, (Scheme 1, mechanism 1), with a slope corresponding to the second order rate constant k_{inact}/K_I ($6.0 \text{ M}^{-1}\text{s}^{-1}$).

Fig. 6. Kinetic evaluation of DUSP5 PD inhibition with eosin Y.

DUSP5 PD activity, measured as the generation of p -nitrophenolate product from p NPP substrate in assay buffer (a) without DTT, and (b) with 1 mM DTT, was monitored over a two hour period in the presence of 0 – 100 μM eosin Y. Each condition was fitted to equation 3, represented as solid lines, from which the apparent first order rate constants for inhibition k_{obs} were obtained at each eosin Y concentration. (c) k_{obs} values generated from –DTT and +DTT experiments were replotted as a function of inhibitor concentration. The hyperbolic line, descriptive of a two-step irreversible inactivation mechanism of DUSP5 PD by eosin Y (Scheme 1, mechanism 2) in the absence of DTT, represents a nonlinear least-squares fit to equation 4, yielding values for k_{inact} , K_I , and k_{inact}/K_I equal to $2.0 \times 10^{-4} \text{ s}^{-1}$, $4.3 \times 10^{-5} \text{ M}$, and $6.6 \text{ M}^{-1}\text{s}^{-1}$, respectively. The straight line through the +DTT data constrained through the origin represents a linear least-squares fit describing a single-step mechanism for the irreversible inactivation of DUSP5 PD, (Scheme 1, mechanism 1), with a slope corresponding to the second order rate constant k_{inact}/K_I ($1.0 \text{ M}^{-1}\text{s}^{-1}$).

Fig. 7. Competitive inhibition of DUSP5 PD by erythrosin B.

Initial DUSP5 PD velocities were measured over a range of substrate (pNPP, 3 - 81 mM) and erythrosin B (0 - 30 μM) concentrations with 1 mM DTT in the assay buffer. (a) The data were fitted to the model equation for competitive inhibition (equation 5) resulting in estimated V_{max} , K_m , and K_i values ($\pm$ SE) of $0.66 \pm 0.02 \mu\text{M}\cdot\text{min}^{-1}$, $11.57 \pm 1.02 \text{ mM}$, and $10.87 \pm 1.21 \mu\text{M}$, respectively. (b) Double reciprocal plot of the initial velocity data, indicating a slope but no y-intercept (V_{max}) effect.

Fig. 8. Noncompetitive inhibition of DUSP5 PD by Rose Bengal.

Initial DUSP5 PD velocities were measured over a range of substrate (pNPP , 3 – 27 mM) and rose bengal (0 – 3.5 μM) concentrations following 15 minute incubations in the dark or exposed to light. The data were fitted to the model equation for noncompetitive inhibition (equation 6) having a single K_i value (panels a and b), and fitted to the model equation for noncompetitive inhibition having two K_i values (equation 7, panels c and d). Double reciprocal plots (not shown) indicated both slope and intercept effects. The single K_i noncompetitive inhibition model K_i values are $2.27 \pm 0.14 \mu\text{M}$ and $0.54 \pm 0.03 \mu\text{M}$ for the

dark and light incubations, respectively. The two K_i noncompetitive inhibition model K_{is} values are $0.35 \pm 0.04 \mu\text{M}$ and $0.08 \pm 0.00 \mu\text{M}$, and the K_{ii} values $2.25 \pm 0.10 \mu\text{M}$ and $0.55 \pm 0.02 \mu\text{M}$, for the dark and light incubations, respectively (additional kinetic information reported in Table S1).

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Fig. 9. Spectroscopic characterization of the Rose Bengal-DUSP5 PD complex dissociation constant (K_d).

(a, d) Rose bengal (6.5 μ M) absorption spectrum from 450 to 650 nm before and after titration with 0.5, 1.0, 1.5, 2.0, and 2.5 μ M DUSP5 PD (a) without and (d) with 1 mM DTT in the assay buffer. (b, e) Rose Bengal difference spectra following subtraction of the spectrum without DUSP5 PD from the buffer sample spectra containing DUSP5 PD. (c, f) DUSP5 PD protein fraction bound to rose bengal plotted against the total sample DUSP5 PD concentration. The lines represent a nonlinear least-squares model fit (equation 8) to the data, yielding average K_d estimates of $0.69 \pm 0.17 \mu$ M in the absence of DTT and $0.81 \pm 0.56 \mu$ M in the presence of DTT (additional details provided in Table S2).

Fig. 10. Merbromin molecular docking.

(a) Electrostatic potential surface of DUSP5 PD, showing location of the two positively charged binding pockets. The active site contains the catalytic cysteine residue. (b) Merbromin in its lowest energy docking pose, spanning the two DUSP5 binding pockets. (c) Rose bengal in its lowest energy docking pose, binding predominantly in the secondary binding site pocket, close to R213 and R214.

Scheme 1: Irreversible enzyme inhibition mechanisms.

Mechanism 1. Single-step inactivation mechanism. Enzyme inactivation is linearly related to inhibitor concentration. Mechanism 2. Two-step inactivation mechanism. The initial step involves the reversible binding of the inhibitor to the enzyme, followed by an irreversible formation of an inactive enzyme-inhibitor complex.

Potential Kinetic Mechanisms for Inhibition

Table 1.

Effect of incubation time on IC₅₀ values (mean ± SD, μM) for selected test compounds against DUSP5 PD activity.

Compound	Without DTT			With DTT		
	0 min	30 min	60 min	0 min	30 min	60 min
<i>Rose Bengal; (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein)</i>	2.9 ± 0.2	2.6 ± 0.4	2.3 ± 0.4	2.7 ± 0.4	2.6 ± 0.5	2.4 ± 0.6
<i>Merbromin; (2',7'-dibromo-5'-[hydroxy-mercurio]-fluorescein)</i>	16.1 ± 0.7	6.5 ± 0.2	4.1 ± 0.1	30.9 ± 0.9	23.6 ± 0.6	19.7 ± 0.6
<i>Erythrosin B; (2',4',5',7'-tetraiodofluorescein)</i>	18.5 ± 0.3	16.0 ± 0.3	14.0 ± 0.3	19.1 ± 0.4	17.2 ± 0.3	15.8 ± 0.3
<i>Eosin Y; 2',4',5',7'-tetrabromofluorescein)</i>	> 100	63.3 ± 6.9	37.6 ± 4.5	> 100	> 100	> 100
<i>2',7'-dibromofluorescein</i>	268.1 ± 22.7	90.9 ± 4.5	44.7 ± 2.2	>300	>300	>300
<i>2',7'-dichlorofluorescein</i>	241.2 ± 5.3	205.5 ± 4.6	179.7 ± 4.5	238.1 ± 6.4	216.7 ± 4.8	200.3 ± 3.1
<i>Fluorescein</i>	> 300	> 300	> 300	> 300	> 300	> 300

Table 2.

Kinetic parameters for irreversible DUSP5 PD enzyme inactivation.

Compound	DTT	k_{inact}/K_I ($\text{M}^{-1}\text{s}^{-1}$)	Inhibitor Mechanism
<i>Merbromin</i>	–	47.08 ± 4.66	two-step
	+	6.22 ± 0.74	single-step
<i>Erythrosin B</i>	–	4.65 ± 0.71	single-step
	+	4.13 ± 0.13	single-step
<i>Eosin Y</i>	–	6.06 ± 2.43	two-step
	+	0.90 ± 0.13	single-step
<i>2',7'-dibromofluorescein</i>	–	2.40 ± 0.48	single-step
	+	0.84 ± 0.07	single-step
<i>2',7'-dichlorofluorescein</i>	–	0.41 ± 0.06	single-step
	+	0.14 ± 0.02	single-step

Table 3

Electrostatic potential surfaces and frontier orbital isosurfaces/energies for the xanthene dyes used in this study.

Xanthene Dye	Electrostatic Potential	LUMO	HOMO
Fluorescein		E (LUMO) = 4.04 eV	E (HOMO) = 1.06 eV
2',7'-dichlorofluorescein		E (LUMO) = 3.65 eV	E (HOMO) = 0.70 eV
2',7'-dibromofluorescein		E (LUMO) = 3.54 eV	E (HOMO) = 0.57 eV
Eosin Y		E (LUMO) = 3.21 eV	E (HOMO) = 0.25 eV
Erythrosin B		E (LUMO) = 3.07 eV	E (HOMO) = 0.06 eV
Merbromin		E (LUMO) = 3.31 eV	E (HOMO) = 0.32 eV
Rose Bengal		E (LUMO) = 2.55 eV	E (HOMO) = -0.22 eV