Slow-Binding Inhibition of Human Prostaglandin Endoperoxide Synthase-2 with Darbufelone, an Isoform-Selective Antiinflammatory Di-*tert*-butyl Phenol

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ABSTRACT: The antiinflammatory agent darbufelone, ((*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl] methylene]-2-imino-4-thiazolidinone, methanesulfonate salt), was discovered as a dual inhibitor of cellular prostaglandin and leukotriene production. To study the mechanism of action of this drug, we expressed human prostaglandin endoperoxide synthase-1 (PGHS-1) and PGHS-2 and purified the recombinant enzymes using buffers that contain octylglucoside. In cyclooxygenase assays following a 15-min incubation of enzyme with inhibitor, darbufelone potently inhibits PGHS-2 ($IC_{50} = 0.19\ \mu M$) but is much less potent with PGHS-1 ($IC_{50} = 20\ \mu M$). Interestingly, when the assay buffer contains traces of Tween 20 (0.0001%), darbufelone appears inactive with PGHS-2 due to a detergent interaction that is detectable by absorption spectroscopy. We therefore used octylglucoside, which does not affect darbufelone in this way, in place of Tween 20 in our PGHS buffers. Inhibition of PGHS-2 with darbufelone is time dependent: with no preincubation, darbufelone is a weak inhibitor ($IC_{50} = 14\ \mu M$), but after a 30-min incubation it is 20-fold more potent. Plots of PGHS-2 activity vs preincubation time at various darbufelone concentrations reach a plateau. This finding is inconsistent with irreversible or one-step slow-binding inhibition. A two-step slow-binding inhibition model is proposed in which the E·I complex ($K_i = 6.2 \pm 1.9$ to $14 \pm 1\ \mu M$) slowly transforms ($k_5 = 0.015-0.030\ s^{-1}$) to a tightly bound E*·I form with $K_i^* = 0.63 \pm 0.07\ \mu M$ and $k_6 = 0.0034\ s^{-1}$. In steady-state kinetics inhibition experiments performed with no preincubation, we find that darbufelone is a noncompetitive inhibitor of PGHS-2 ($K_i = 10 \pm 5\ \mu M$). Darbufelone quenches the fluorescence of PGHS-2 at 325 nm ($\lambda_{ex} = 280$ nm) with $K_d = 0.98 \pm 0.03\ \mu M$. The PGHS substrate, arachidonate, and various cyclooxygenase inhibitors do not alter this binding affinity of darbufelone but a structural analogue of darbufelone competes directly for binding to PGHS-2. Di-*tert*-butyl phenols such as darbufelone may inhibit PGHS-2 by exploiting a previously unrecognized binding site on the enzyme.

The cyclooxygenase activity of the heme-containing enzyme, prostaglandin endoperoxide synthase (PGHS,[1] EC 1.14.99.1), converts arachidonic acid to the endoperoxy-hydroperoxide, prostaglandin (PG) G_2. The PGHS peroxidase activity reduces PGG_2 to the endoperoxy-alcohol, PGH_2, which is converted by specific synthases to bioactive and inflammatory PGs and thromboxanes. In addition, 5-lipoxygenase (5-LO, EC 1.13.11.34) converts arachidonic acid first to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid which it then transforms to the epoxide, leukotriene (LT) A_4. LTA_4 is metabolized to the inflammatory mediator, LTB_4. Although arthritis and inflammation therapy has focused on inhibiting PGHS, a dual inhibitor of PGHS and 5-LO (*1*) may be advantageous because effects of PGs, thromboxanes, and LTs are all attenuated.

Two PGHS isoforms exist. PGHS-1 is expressed at a low, constitutive level in most tissues whereas expression of PGHS-2 is highly induced by inflammatory signals (*2*−*7*), at sites undergoing an inflammatory response (*8*−*10*), and in various cancers (*11*). An early hypothesis was that selective PGHS-2 inhibitor drugs would have few side effects. PGHS-1 could still produce protective PGs but the synthesis of proinflammatory eicosanoids by PGHS-2 in inflamed regions would be blocked (*12*, *13*). Reports of PGHS-2 inhibitors that show little or no gastric irritation upon administration support this hypothesis (*13*, *14*). Several classes of PGHS-2 inhibitors have been described: methanesulfonanilides such as NS-398 (*13*), tricyclic compounds DuP697 (*15*), SC-58125 (*16*), and celecoxib (*17*, *18*), and oxicams such as meloxicam (*19*, *20*). NS-398, DuP697, and celecoxib combine time-dependent, slow-binding inhibition of PGHS-2 (*15*, *18*, *21*) with no inhibition or weak, rapid equilibrium inhibition of PGHS-1. An early report indicated that reversible slow-binding inhibitors could interact with their target in a slow one-step mechanism or through a two-step mechanism in which the equilibrium of the second step is very slow (*22*). After many examples of slow-binding

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[1] Abbreviations: LT, leukotriene; PG, prostaglandin; PGHS, PG endoperoxide synthase; apoPGHS, PGHS without bound heme; holoPGHS, PGHS reconstituted with heme; darbufelone, ((*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methylene]-2-imino-4-thiazolidinone, methanesulfonate salt); TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)-aminomethane; MOI, multiplicity of infection; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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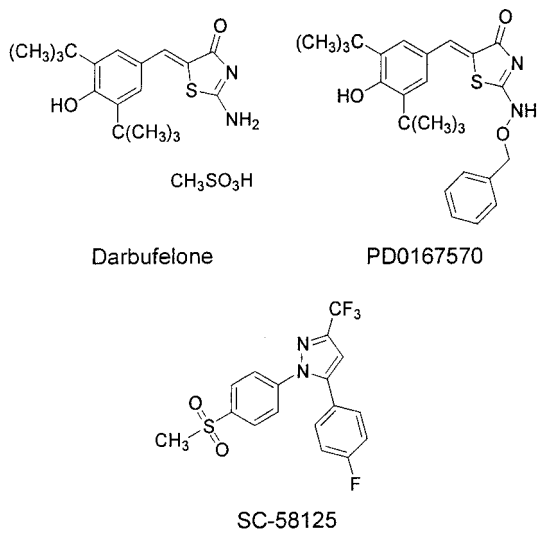


FIGURE 1: Chemical structures of the PGHS-2 inhibitors used in these studies. Darbufelone ((*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methylene]-2-imino-4-thiazolidinone, methanesulfonate salt), also known as PD0136095-73 and CI-1004, and PD0167570 ((*Z*)-2-benzyloxyamino-5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-thiazol-4-one) are PGHS inhibitors from Pfizer. SC-58125 (1-[(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole) is a selective PGHS-2 inhibitor from Searle/Monsanto.

inhibition had appeared, it was suggested that most slow-binding inhibitors followed the two-step model (*23*).

Darbufelone ((*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methylene]-2-imino-4-thiazolidinone, methanesulfonate salt; also known as CI-1004 and PD0136095-73) (Figure 1) is a novel antiinflammatory di-*tert*-butyl phenol that was identified as a dual inhibitor of cellular $PGF_{2\alpha}$ and LTB_4 production (*24*, *25*). Darbufelone is orally active and nonulcerogenic in animal models of inflammation and arthritis (*24*, *25*). Although several antiinflammatory di-*tert*-butyl phenols have been reported (*26*−*29*), no mechanistic investigation of this structural class has appeared.

In our studies of inhibition of human PGHS isoforms, we find that the di-*tert*-butyl phenol darbufelone is a slow-binding, noncompetitive inhibitor of PGHS-2 that is 100-fold less potent with PGHS-1. We note a dramatic loss of potency of darbufelone with PGHS-2 when the assay buffer contains trace levels of Tween 20, a detergent that is often present in buffers used with PGHS. Whereas changes in inhibitor fluorescence have previously been monitored to detect binding to PGHS (*30*−*32*), addition of darbufelone to PGHS-2 quenches the protein fluorescence, which allows us to estimate the binding affinity. Results of ligand competition binding experiments indicate that darbufelone binds PGHS-2 at a site that is distinct from that where arachidonate and known cyclooxygenase inhibitors bind. Darbufelone, as well as other di-*tert*-butyl phenols, may inhibit PGHS-2 by exploiting a previously unrecognized, novel binding site on the enzyme.

EXPERIMENTAL PROCEDURES

Materials. Tween 20 (10%, protein grade) and octyl-β-D-glucopyranoside (octylglucoside, Ultrol grade) were from Calbiochem. [1-^{14}C]Arachidonic acid was from Amersham while sealed ampules of unlabeled arachidonic acid were from NuChek Prep (Elysian, MN). *Spodoptera frugiperda* (*Sf*9) cells, pFASTBAC1, and Sf-900 II serum-free insect cell culture medium were products of Life Technologies. Inhibitor test compounds were synthesized by the Pfizer Medicinal Chemistry Department and were >99% pure. Iron-(III) protoporphyrin IX chloride (heme) from Porphyrin Products was prepared as a 2 mM stock in DMSO and stored at −20 °C. TMPD was from Sigma. The bicinchoninic acid protein assay kit (Pierce) was used to quantify the level of protein in samples using bovine serum albumin as the standard. Ultrafiltration materials were from Amicon.

Expression and Purification of Recombinant Human PGHS-1 and -2. PGHS-1 (*33*) was expressed using the BacToBac baculovirus expression system according to the manufacturer's protocol (Life Technologies). *Sf*9 cells growing at 27 °C in spinner flasks in Sf-900 II serum-free medium (0.5 L) containing 50 units/mL penicillin and 50 μg/mL streptomycin were infected at a multiplicity of infection (MOI) of 0.5 and a cell density of 2×10^6 cells/mL. Cells were harvested by centrifugation 72 h postinfection and the cell pellets were stored at −80 °C until they were used. A PGHS-2 clone was isolated by reverse transcriptase-PCR from mRNA produced by phorbol myristate acetate-stimulated human umbilical vein endothelial cells (*6*). The PGHS-2 coding sequence was subcloned into pBlueBac III (Invitrogen) for baculovirus expression in *Sf*9 cells. Transfected cells were subjected to plaque purification and three sequential steps to reach a virus titer of at least 10^8 plaque forming units/mL.

Enzyme Purification. All steps were carried out at 4 °C. PGHS in various samples was located by monitoring the H_2O_2 peroxidase activity in a TMPD-linked colorimetric assay (*34*, *35*). Recombinant human PGHS-1 and PGHS-2 were purified from baculovirus-infected *Sf*9 cells as described (*36*−*39*) with some modifications. PGHS-1 was extracted from the membrane fraction with 0.75% (v/v, 6.1 mM) Tween 20. After ultracentrifugation, the soluble protein was applied to a DEAE-Sepharose column in 20 mM Tris-HCl buffer (pH 8.0) (buffer A) containing 0.8% (27.4 mM) octylglucoside (buffer B). The column was washed exhaustively with 10 column volumes of buffer B to remove Tween 20 and was eluted with a linear gradient of NaCl to 0.3 M. Fractions containing PGHS-1 were pooled and ultrafiltered. The concentrate was applied to and eluted from a Sephacryl S-300 column in buffer B. Fractions containing PGHS-1 were pooled and applied to a Q-Sepharose column in buffer B which was eluted with a linear gradient of NaCl to 0.5 M. Fractions containing PGHS-1 were pooled and ultrafiltered. In initial trials, PGHS-2 also was extracted with Tween 20 (0.5%). After ultracentrifugation, the soluble protein was applied to a DEAE-Sepharose column in buffer A plus 0.1% Tween 20. The column was washed with this buffer, then eluted with a linear gradient of NaCl to 0.5 M. Fractions containing PGHS-2 were pooled and ultrafiltered. The concentrate was applied to and eluted from a Sephadex G-200 column in 20 mM triethylamine-HCl buffer (pH 8.5) plus 0.1% Tween 20. In subsequent trials, PGHS-2 was solubilized instead with 1.3% (w/v, 450 mM) octylglucoside. The soluble protein obtained after ultracentrifugation was applied to a DEAE-Sepharose column in buffer B. The column was washed with buffer B and eluted with a linear gradient of NaCl to 0.3 M. Fractions containing PGHS-2

were pooled and ultrafiltered. The concentrate was applied to and eluted from a Sephacryl S-300 column in buffer B. Fractions containing apoPGHS-2 were pooled and concentrated. All purified PGHS preparations were obtained as heme-free apoenzymes and were stored at 4 °C. Unless otherwise noted, PGHS holoenzymes were reconstituted by incubating the apoenzyme with 1.5 equiv of heme immediately prior to use.

Radiometric Cyclooxygenase Assays. The inhibition of cyclooxygenase activity was assessed by monitoring the PGHS-catalyzed conversion of [1-^{14}C]arachidonate to polar reaction products. Incubations were carried out at room temperature (22−23 °C) and contained 20 mM Tris-HCl buffer (pH 7.4), 2 mM epinephrine, 0.1 μM heme, 23 μM arachidonate, and inhibitor in 5% DMSO. Test compounds were diluted in buffer to two times the final concentration (in 40 μL) before 20 μL of PGHS were added (30 nM final concentration) and allowed to incubate for 15 min. One minute after 20 μL of [1-^{14}C]arachidonate was added, the reaction was stopped by the addition of 150 μL of quench solution [methanol:tetrahydrofuran:acetic acid (40:40:20), plus 0.5 mg/mL triphenylphosphine]. Polar reaction products in 150 μL of quenched reaction mixture were separated from substrate by reversed-phase HPLC with isocratic elution at 1.1 mL/min using a 4.6 × 50 mm, 300 Å, 5 μm C_{18} Dynamax column (Rainin). The mobile phase was methanol:tetrahydrofuran:water:acetic acid (55:25:20:0.1). A Flo-One Beta Series A-200 radio-chromatography detector (Packard) was used to monitor and quantify eluted radioactive components. The blank conversion value was subtracted from each sample and the percent of control cyclooxygenase activity was calculated using eq 1.

$$\%\ \text{control activity} = (\text{inhibited rate}/\text{control rate}) \times 100 \quad (1)$$

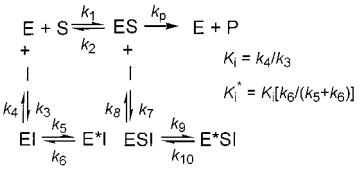
IC_{50} values were calculated using the KaleidaGraph software package (Synergy; Reading, PA) by fitting the percent of control activity vs inhibitor concentration data to eq 2 that describes a sigmoid inhibition pattern, where max is

$$\%\ \text{control activity} = \text{max}/[1 + ([\text{I}]/\text{IC}_{50})^{\text{slope}}] \quad (2)$$

the upper limit of the percent activity value, [I] is the inhibitor concentration, IC_{50} is the concentration of inhibitor where the reaction rate is 50% inhibited relative to the control, and slope is the slope of the curve at its inflection point.

Spectrophotometric Cyclooxygenase Assays. Cyclooxygenase activity was also quantified by monitoring the oxidation of the peroxidase cosubstrate TMPD at 610 nm during the (rate limiting) oxidation of arachidonic acid (*34*, *35*). HoloPGHS in 20 mM Tris-HCl buffer (pH 7.4) was incubated for specified times at room temperature with inhibitor or DMSO vehicle. After the preincubation phase, enzymatic reactions were initiated by adding 25 μM arachidonic acid and 100 μM TMPD. Initial rates of product formation were monitored using a microplate reader thermostated at 25 °C. This temperature, rather than 37 °C, was used to allow for more accurate measurement of initial velocities with less interference from the turnover-dependent inactivation of the cyclooxygenase activity. The initial rate of TMPD oxidation over the first 30 s was converted to percent of control data using eq 1. The IC_{50} values determined in this colorimetric TMPD-linked assay agree with those obtained using the radiometric substrate conversion assay.

Scheme 1: Proposed Mechanism for Slow-Binding, Noncompetitive Inhibition of PGHS-2 by Darbufelone



Steady-State Kinetics of Inhibition of PGHS-2 with Darbufelone. The effect of darbufelone on the cyclooxygenase activity of PGHS-2 was determined in assays with no enzyme−inhibitor preincubation. HoloPGHS-2 (30 nM final concentration) was added to reaction mixtures that contained 20 mM Tris-HCl buffer (pH 7.4), 100 μM TMPD, and varying levels of arachidonic acid (0−60 μM) and darbufelone (0−30 μM). The cyclooxygenase activity was measured by monitoring the oxidation of TMPD at 610 nm using a microplate reader.

Time Dependence of Inhibition of PGHS-2 with Darbufelone. The inhibition of PGHS-2 activity was assessed using the spectrophotometric assay by varying the time of preincubation of PGHS-2 with darbufelone before initiating reactions with 25 μM arachidonic acid and 100 μM TMPD. Incubation mixtures contained 20 mM Tris-HCl buffer (pH 7.4), 5% DMSO (without or with 0.001−30 μM darbufelone), and 20 nM holoPGHS-2. Seventy titrations using preincubation times of 15−1800 s were carried out in order to provide sufficient data to characterize the time course of the potency shift. The mean IC_{50} data plotted as a function of preincubation time were fit to a model for an exponential decay to a finite value according to eq 3, where $IC_{50,init}$ and $IC_{50,eq}$ are the potency with no preincubation and at equilibrium, respectively, k_{obs} is the

$$\text{IC}_{50} = \text{IC}_{50,\text{init}}[\exp(-k_{\text{obs}}t)] + \text{IC}_{50,\text{eq}} \quad (3)$$

observed exponential decay rate constant, and t is the preincubation time.

The kinetics of inhibition of PGHS-2 with darbufelone were also analyzed by varying the preincubation time and monitoring the remaining activity in the colorimetric cyclooxygenase assay. At each inhibitor concentration, the percent of control activity data were plotted as a function of preincubation time and fit to eq 4 in order to estimate values for k_{obs}, the pseudo-first

$$\%\ \text{control activity} = 100[\exp(-k_{\text{obs}}t)] + \text{baseline} \quad (4)$$

order rate constant for the inhibition process; t is the preincubation time and baseline is the percent of control activity at equilibrium. The k_{obs} values were plotted as a function of darbufelone concentration and fit to eq 5 (assuming $k_6 \cong 0$) where $k_{obs,max}$ is the rate constant for

$$k_{\text{obs}} = k_{\text{obs,max}}[\text{I}]/(K_{\text{i}} + [\text{I}]) \quad (5)$$

the E·I → E*·I conversion (see Scheme 1), K_i is the inhibition constant for darbufelone, and [I] is the inhibitor concentration

(*15*, *18*, *21*). The percent of control activity remaining at equilibrium for each inhibitor concentration was plotted as a function of inhibitor concentration and fit to eq 6 for a decreasing hyperbolic function

$$\% \text{ control activity at equilibrium} = 100[1 - ([I]/(K_i^* + [I]))] \quad (6)$$

where [I] is the darbufelone concentration and K_i^* is the overall dissociation constant for release of darbufelone from the E*·I complex.

Absorption Spectroscopy of Tween 20/Darbufelone Interactions. A Hewlett-Packard 8452A diode array spectrophotometer was used with the cuvette holder thermostated at 25 °C (i.e., the temperature that was used in the inhibition assays). Darbufelone (2 mM in methanol) was diluted to 10 μM in 20 mM potassium phosphate buffer (pH 7.4) and its initial absorption spectrum was recorded. The spectrum was recorded 15 min after adding either 0.0001% (0.8 μM) Tween 20 or 0.0005% (17 μM) octylglucoside from aqueous 0.1% detergent solutions.

Static Fluorescence Quenching Binding Titrations. A Perkin-Elmer LS-50B luminescence spectrometer equipped with a cuvette holder thermostated at 25 °C was used. Fluorescence emission (10 nm slit) was recorded at 50 nm/min upon excitation at 280 nm (2.5 nm slit). Ligands were added directly into the fluorescence cuvette (path lengths: ex, 4 mm; em, 10 mm) from DMSO stock solutions; the final DMSO concentration was <1% (v/v). The fluorescence at 325 nm data were fit to eq 7 for a hyperbolically decreasing one-site binding isotherm

$$F_{325} = \Delta F_{325,max}[1 - ([L]/(K_d + [L]))] + F_{325,final} \quad (7)$$

where $\Delta F_{325,\,max}$ is the total change in PGHS-2 fluorescence at 325 nm at saturation, [L] is the total concentration of ligand added, K_d is the dissociation constant of the ligand, and $F_{325,final}$ is the fluorescence of the enzyme at ligand saturation.

In competition binding experiments, apoPGHS-2 alone or with 5 μM arachidonic acid and holoPGHS-2 with 40 μM arachidonic acid were titrated with darbufelone as was holoPGHS-2 that was acetylated by incubation with acetyl salicylate (*30*). The competitive potential of 10 μM ibuprofen, which binds in the cyclooxygenase active site but does not quench PGHS-2 fluorescence (*30*), was also tested. A selective PGHS-2 inhibitor, SC-58125, and an analogue of darbufelone, PD0167570, were also tested for their ability to compete with darbufelone. The apparent K_d of darbufelone was determined at several fixed concentrations of these ligands. The $K_{d,app}$ for darbufelone was plotted vs the ligand concentration and the data were fitted by linear regression to eq 8

$$K_{dA,app} = K_{dA} + K_{dA}[B]/K_{dB} \quad (8)$$

which describes the variation in K_d of ligand A (darbufelone) caused by ligand B where $K_{dA,app}$ is the apparent affinity of darbufelone at the concentration [B] of ligand B (i.e., PD0167570 or SC-58125), K_{dA} is the affinity of darbufelone in the absence of ligand B, and K_{dB} is the affinity of ligand B for PGHS-2 in the absence of ligand A.

RESULTS

Expression and Purification of Recombinant Human PGHS-1 and -2. The level of PGHS-1 activity expressed was equal at MOI values from 0.1 to 5 and was highest from 48 to 72 h postinfection, as assessed in [^{14}C]arachidonate conversion assays of cell-free extracts of infected *Sf*9 cells. Thus, a harvest time of 72 h and an MOI of 0.5 were used for PGHS-1 expressions. For PGHS-2, a high level of activity was achieved over a broad range of time after infection and an MOI range of 0.1−4. An MOI of 4 and a harvest time of 72 h were used for PGHS-2 expression. These parameters are similar to those used previously for PGHS (*36*−*39*).

PGHS-1 was poorly extracted from the membrane fraction using Tween 20, dodecyl maltoside, octylglucoside, or CHAPS. After detergent treatment of the *Sf*9 cell lysate and ultracentrifugation, ~90% of the PGHS-1 antibody-responsive protein typically remained in the pellet. However, because Tween 20 yielded the greatest amount of PGHS-1 activity, this detergent was used to initially extract PGHS-1 from the microsomal fraction but was replaced with octylglucoside in subsequent steps. The PGHS-1 that remained in the supernatant after detergent solubilization was only the highest molecular weight (i.e., most highly glycosylated) PGHS-1 detected in cell-free lysates (not shown). From 2.5 L of *Sf*9 cell culture, we purified ~1 mg of PGHS-1 to ~80% homogeneity as assessed by SDS−PAGE (not shown). Whereas pure PGHS-1 was not obtained in substantial amounts, PGHS-2 was readily solubilized and purified to ~95% purity as judged by SDS−PAGE (not shown) using buffers containing Tween 20 or octylglucoside. We obtained ~8 mg of pure PGHS-2/L of *Sf*9 cell culture. As reported by others (*36*, *38*, *39*), PGHS-2 appeared as three glycoforms on SDS−PAGE (not shown).

Inhibition of PGHS-1 and -2 with Darbufelone. The effect of darbufelone on the cyclooxygenase activity of PGHS-1 and -2 was first assessed after a 15-min preincubation of enzyme with inhibitor. IC_{50} values were identical in both the radiometric and spectrophotometric assays. Table 1 summarizes the IC_{50} values of darbufelone with PGHS-2 in the radiometric substrate conversion assay (i.e., with no TMPD) and in the TMPD-linked arachidonic acid conversion assay after different preincubation times. It is evident that TMPD does not change the potency of darbufelone.

Table 1: TMPD Does Not Affect the Potency of Darbufelone with PGHS-2

preincubation time (min)	IC_{50} of darbufelone (μM) using different assays[a]	
	no TMPD[b]	0.1 mM TMPD[c]
1	4.8	4.5
2	2.5	2.8
15	1.1	0.5

[a] IC_{50} values are the average of 2−10 determinations. [b] Using the [^{14}C]arachidonic acid conversion radiometric assay. [c] Using the arachidonic acid TMPD-linked colorimetric assay.

In terms of isoform selectivity, darbufelone is a potent PGHS-2 inhibitor (IC_{50} = 0.19 μM) but is much less effective against PGHS-1 (IC_{50} = 20 μM) (Figure 2) when the assay buffer contains 0.0005% octylglucoside. At low levels, darbufelone appears to stimulate the cyclooxygenase activity of PGHS-1 (eq 2, max = 125%) but not PGHS-2 (eq 2, max = 100%). Interestingly, when the assay buffer contains 0.0001% (0.8 μM) Tween 20, darbufelone appears inactive with PGHS-2 (Figure 2, triangles).

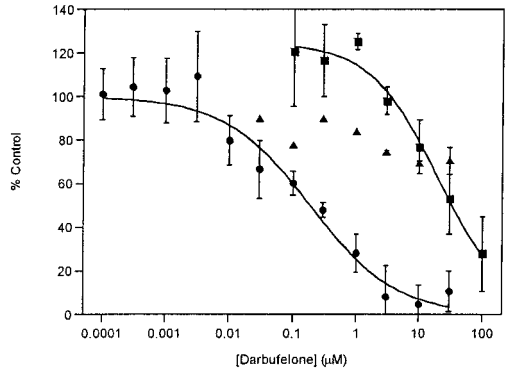


FIGURE 2: Inhibition of purified recombinant human PGHS-1 and PGHS-2 with darbufelone and the adverse effect of Tween 20 on the potency of darbufelone with PGHS-2. The inhibition of PGHS-1 and PGHS-2 with darbufelone was assessed by radiometric [^{14}C]-arachidonate conversion assays or spectrophotometric TMPD oxidation assays. Results using either assay were the same. In the experiments shown, reactions were initiated with arachidonate after a 15-min incubation of enzyme with inhibitor. Data are the mean ± SD of two or three determinations. The assay buffer used with PGHS-1 (■) and PGHS-2 (●) contained 0.0005% octylglucoside. As derived from the fit of the data to eq 2, darbufelone inhibits PGHS-2 with IC_{50} = 0.19 μM and inhibits PGHS-1 with IC_{50} = 20 μM. Darbufelone stimulates by 25% the activity of PGHS-1, but not PGHS-2. Interestingly, darbufelone is ineffective with PGHS-2 when the assay buffer contains just 0.0001% (0.8 μM) Tween 20 (▲).

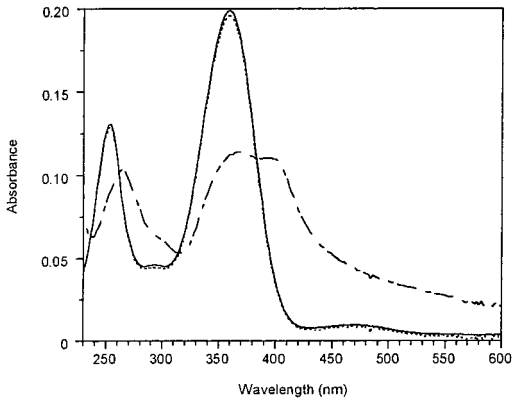


FIGURE 3: Tween 20, but not octylglucoside, alters the absorption spectrum of darbufelone. The absorption spectrum of 10 μM darbufelone in 20 mM potassium phosphate buffer (pH 7.4) is shown (—); the spectrum is unchanged over 15 min. Likewise, 15 min after addition of 0.0005% octylglucoside, the spectrum of darbufelone remains unchanged (- - -). However, 15 min after adding 0.0001% Tween 20 to a darbufelone solution (— —), the absorption at 360 nm decreases dramatically while that at 405 nm as well as the baseline at long wavelengths increases.

Absorption Spectroscopy of Detergent−Inhibitor Interactions. To probe the basis for this unexpected and adverse detergent effect, we assessed the stability of darbufelone in detergent solutions. We selected detergent concentrations equal to those used in the inhibition assays shown in Figure 2 and a 15-min incubation to mimic the preincubation used. With either no detergent or 0.0005% (17 μM) octylglucoside added, the absorption spectrum of 10 μM darbufelone is stable for 15 min (Figure 3). However, after adding 0.0001% (0.8 μM) Tween 20 the darbufelone spectrum is dramatically altered. After 15 min, the absorption of darbufelone had decreased at 360 nm while the absorption at 405 nm and the baseline at long wavelengths increased (Figure 3).

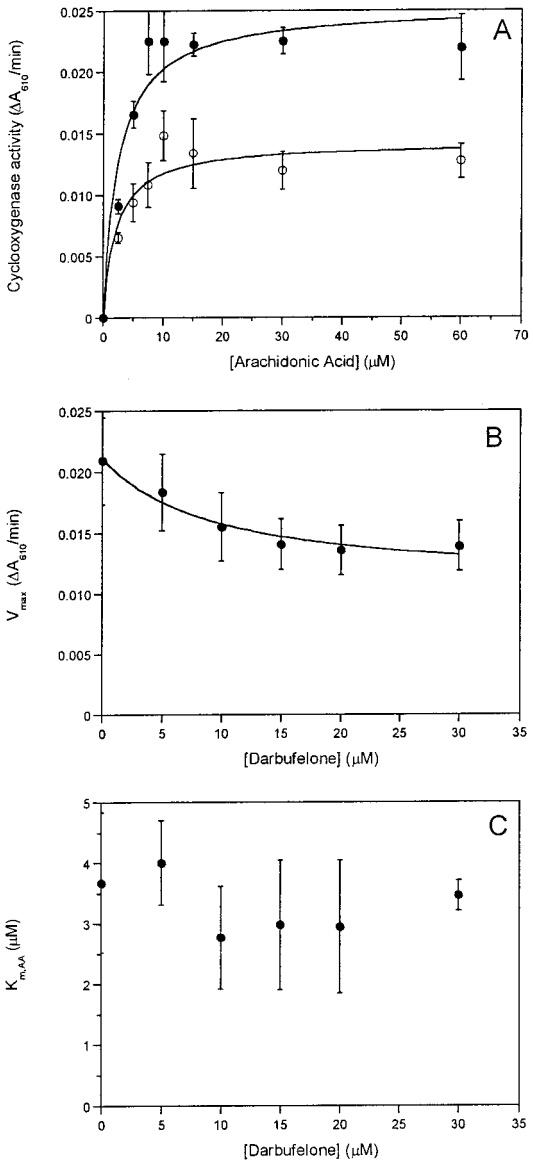


FIGURE 4: Steady-state kinetics of inhibition of PGHS-2 with darbufelone. (A) The cyclooxygenase activity of uninhibited PGHS-2 is plotted as a function of the arachidonic acid concentration (●); V_{max} = 0.025A_{610}/min and K_m = 3.6 ± 1.2 μM. In the presence of 20 μM darbufelone (○), the V_{max} is decreased to 0.014A_{610}/min but K_m is unchanged (K_m = 3.0 ± 1.1 μM). (B) Darbufelone decreases the V_{max} for the initial cyclooxygenase activity of PGHS-2 with K_i = 10 ± 5 μM but has no effect on the K_m of arachidonic acid (Figure 4C). Data shown are the average ± SD from three separate experiments.

Steady-State Mechanism of Inhibition of PGHS-2 by Darbufelone. In these experiments that were carried out with no preincubation, darbufelone appears to be a noncompetitive inhibitor of PGHS-2 (Figure 4A). The V_{max} for cyclooxygenase activity decreases hyperbolically with increasing levels of inhibitor (K_i = 10 ± 5 μM darbufelone) (Figure 4B) whereas the K_m for arachidonic acid is unchanged (K_m = 2.9−4.0 μM) by the presence of darbufelone (Figure 4C).

Time Dependence of Inhibition of PGHS-2 with Darbufelone. The following equilibria (see Scheme 1) occur during preincubation: $E + I \rightleftarrows E{\cdot}I \rightleftarrows E^{*}{\cdot}I$. Reactions are initiated by adding a saturating level of arachidonic acid. The level of activity observed is influenced most by the position of the $E{\cdot}I \rightleftarrows E^{*}{\cdot}I$ equilibrium, which is slow relative

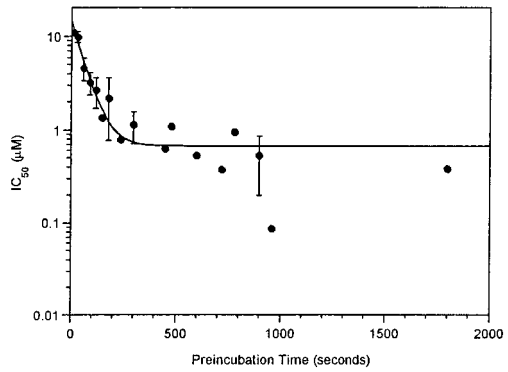


FIGURE 5: Effect of preincubation time on the IC_{50} of darbufelone with PGHS-2. The inhibition of PGHS-2 with 0.001 to 30 μM darbufelone was assessed in cyclooxygenase inhibition assays initiated with 25 μM arachidonic acid and 100 μM TMPD after a 0.25−30 min preincubation. IC_{50} values (mean ± SD) from 70 titrations are plotted. The line drawn is a fit to eq 3 that describes a single-exponential decay of the IC_{50} to a minimum value.

to the E + I ⇄ E·I equilibrium, and is fully achieved only after long preincubation times. Because there is no substrate present during the preincubation phase, no ES or ESI forms and rate constants k_7-k_{10} are not incorporated into these analyses.

The time-dependent decrease in the IC_{50} is fit to a single-exponential decay to a plateau value (Figure 5). Extrapolating the data in Figure 5 back to zero preincubation time shows that darbufelone inhibits PGHS-2 with an $IC_{50} = 14 \pm 1$ μM. After a 30-min (1800 s) preincubation, the mean $IC_{50} = 0.67 \pm 0.17$ μM. Because equilibrium is achieved from both directions and a plateau in the IC_{50} value is observed, k_{obs} in Figure 5 should approximate ($k_5 + k_6$), the sum of the rate constants of the E·I ⇄ E*·I equilibrium. In this case, $k_{obs} = 0.018 \pm 0.002$ s^{-1}.

Slow-Binding Kinetics of Inhibition of PGHS-2 with Darbufelone. The time-dependent decay in cyclooxygenase activity to a finite residual activity level at various concentrations of darbufelone is shown in Figure 6A. The activity data at each darbufelone concentration were fit to single-exponential decay to a baseline activity in order to determine the observed inhibition rate constant, k_{obs}. The plot of k_{obs} vs darbufelone concentration is hyperbolic (Figure 6B), which indicates the presence of a slow rate-limiting step after the inhibitor binding step. As derived from this plot, the inhibition constant for darbufelone, $K_i = 6.2 \pm 1.9$ μM. Since $k_6 \cong 0$, the plateau of Figure 6B provides an estimate for $k_5 = 0.030 \pm 0.004$ s^{-1}, the rate constant for the E·I → E*·I isomerization.

*Determination of K_i**. The finding that PGHS-2 activity plateaus at a finite level after long times of preincubation with darbufelone (Figure 6A) indicates that k_6 indeed has a nonzero value and that the E·I ⇄ E*·I equilibrium is reversible. Thus, a plot of the percent of control activity at equilibrium vs inhibitor concentration (Figure 6C) provides an estimate of the overall dissociation constant, K_i*, for release of darbufelone from the E*·I complex. A fit of data from three experiments to eq 6 indicates $K_i^* = 0.63 \pm 0.07$ μM (Figure 6C), a value that is close to the IC_{50} for darbufelone that is attained at equilibrium (Figure 5). Combining data for darbufelone obtained from several different experimental approaches and the equation $K_i^* = K_i k_6/(k_5 + k_6)$ which was derived previously for slow-binding

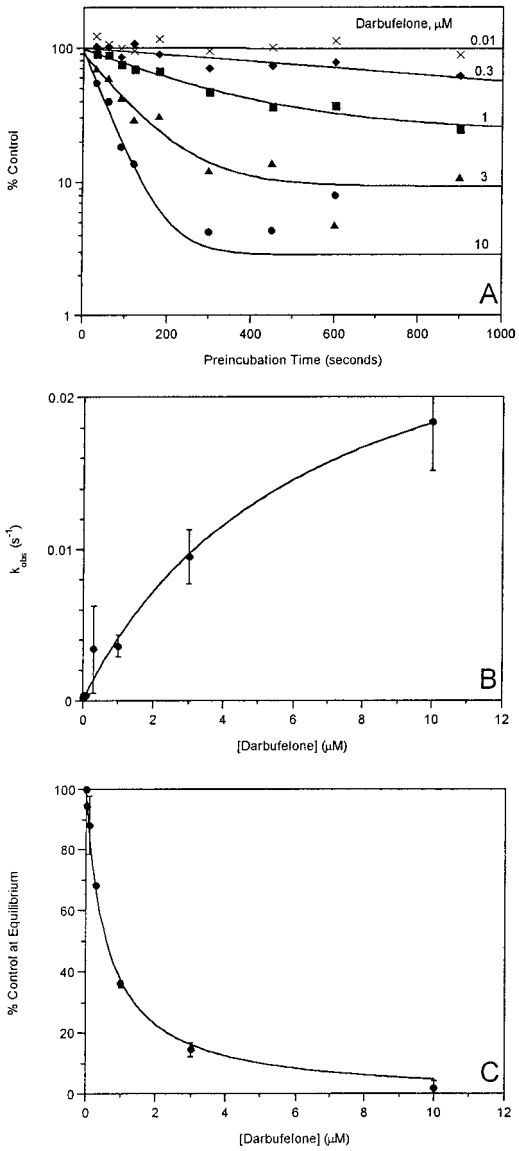


FIGURE 6: Slow-binding kinetics of inhibition of PGHS-2 with darbufelone. (A) The percent of control cyclooxygenase activity remaining after preincubation of PGHS-2 with darbufelone for various times is plotted vs the preincubation time. The data at each darbufelone concentration are fit to eq 4 that describes a single-exponential decay to a plateau residual activity. The data shown are from one experiment that has been repeated five times with similar results each time. (B) Hyperbolic dependence of the observed rate constant, k_{obs}, for inhibition of PGHS-2 on the darbufelone concentration. The k_{obs} values derived from Figure 6A are plotted as a function of darbufelone concentration. Error bars represent the error in k_{obs} derived from the fit of the inhibition data to eq 4. The line drawn is the best fit to eq 5. (C) Determination of K_i* from the plot of the equilibrium cyclooxygenase activity remaining vs darbufelone concentration. The mean ± SD percent of control cyclooxygenase activity remaining at equilibrium (after 900 s preincubation) from three experiments is plotted as a function of darbufelone concentration. The line drawn is a fit of the data to eq 6.

inhibitors (*22*, *23*), we calculate the value for k_6, the rate constant for the E*·I → E·I isomerization (see Scheme 1) to be on the order of 0.0034 s^{-1} (Table 2).

K_d Values of Darbufelone and PD0167570 with PGHS-2. The absorption of darbufelone (Figure 3) is maximal at 359 nm but is minimal at 280 nm. The fluorescence of darbufelone is negligible from 300 to 500 nm ($\lambda_{ex} = 280$ nm,

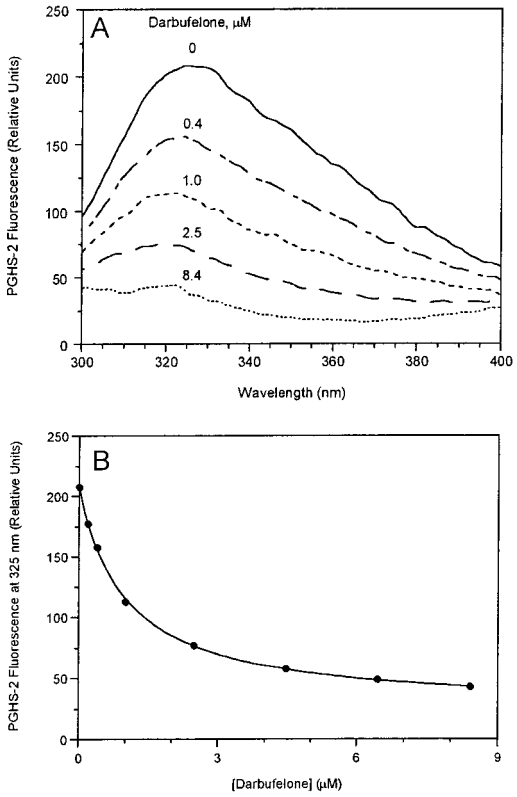


FIGURE 7: Darbufelone quenches PGHS-2 fluorescence. (A) Using an excitation wavelength of 280 nm, holoPGHS-2 (0.1 μM) in 20 mM potassium phosphate buffer (pH 7.4) displays maximum fluorescence at 325 nm. Spectra are baseline-corrected for buffer contribution. The fluorescence emission spectra of PGHS-2 obtained after the addition of various levels of darbufelone are shown. The wavelength of maximum emission of PGHS-2 is somewhat blue-shifted to 318 nm in the presence of saturating levels of darbufelone. (B) Isotherm for binding of darbufelone to PGHS-2. The PGHS-2 fluorescence at 325 nm shown in Figure 7A is plotted as a function of darbufelone concentration. The data are fit to eq 7 that describes a thermodynamic equilibrium binding isotherm with a decreasing function. The $K_d = 0.98 \pm 0.03$ μM darbufelone.

Table 2: Kinetic and Thermodynamic Constants for Inhibition of PGHS-2 with Darbufelone

parameter	method of determination	value
$k_5 + k_6$ (s^{-1})	k_{obs}, Figure 5	0.018 ± 0.002
k_5 (s^{-1})	$k_{obs,max}$, Figure 6B	0.030 ± 0.004
k_6 (s^{-1})	$= (k_5K_i^*)/(K_i - K_i^*)$, data of Figure 6	0.0034
k_5 (s^{-1})	$= k_{obs} - k_6$; k_{obs}, Figure 5 and k_6, Figure 6	0.015
K_i (μM)	IC_{50} at $t = 0$, Figure 5	14 ± 1
K_i (μM)	steady state kinetics, Figure 4B	10 ± 5
K_i (μM)	from Figure 6B	6.2 ± 1.9
K_i^* (μM)	IC_{50} at equilibrium, Figure 5	0.67 ± 0.17
K_i^* (μM)	from Figure 6C	0.63 ± 0.07
K_d (μM)	fluorescence quenching; Figure 7B	0.98 ± 0.03

not shown). As can be seen in Figure 7A, the fluorescence of PGHS-2 is maximal at 325 nm ($\lambda_{ex} = 280$ nm) consistent with previous reports (*30*, *31*). Thus, there is significant overlap between the emission spectrum of the donor, PGHS-2, and the absorption spectrum of the acceptor, darbufelone. To determine the equilibrium dissociation constant for darbufelone, the effect of this inhibitor on the PGHS-2 protein fluorescence was monitored. Addition of darbufelone to PGHS-2 causes a concentration-dependent quenching of the enzyme's fluorescence (Figure 7A). Evidence that

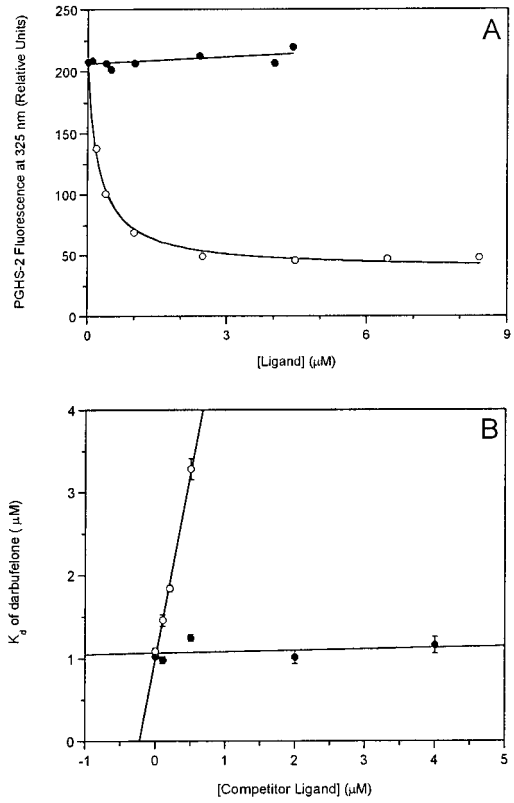


FIGURE 8: Saturable quenching of PGHS-2 fluorescence by PD0167570 but not by SC-58125. (A) The fluorescence emission of 0.1 μM holoPGHS-2 was monitored at 325 nm upon excitation of the sample at 280 nm. The fluorescence data for titration with PD0167570 (○) are fitted to eq 7 that describes a thermodynamic equilibrium binding isotherm with a decreasing function. For PD0167570, $K_d = 0.25 \pm 0.03$ μM. Since SC-58125 does not quench PGHS-2 fluorescence, the data obtained during titration with SC-58125 (●) were arbitrarily fit to a linear function. (B) Competitive effect of PD0167570, but not SC-58125, on the binding affinity of darbufelone with PGHS-2. The apparent K_d of darbufelone with PGHS-2 was determined in fluorescence quenching titrations carried out in the presence of various levels of PD0167570 (○) or SC-58125 (●). The apparent K_d data were fitted by linear regression to eq 8 for a competitive titration. The intercept on the *x*-axis equals $-K_d$ for the competitor, PD0167570 ($K_d = 0.23$ μM, which is in agreement with $K_d = 0.25$ μM determined for PD0167570 in Figure 8A). On the other hand, SC-58125 at up to 40 times its IC_{50} for inhibition of PGHS-2 (i.e., 0.1 μM) has no effect on the affinity of darbufelone.

darbufelone is binding to a specific site on PGHS-2 is provided by the saturable decrease in PGHS-2 fluorescence during the titration (Figure 7B). As derived from the data for darbufelone in Figure 7B, the $K_d = 0.98 \pm 0.03$ μM. This K_d is close to the K_i^* value determined from the minimal IC_{50} value attained at equilibrium (Figure 5) and the kinetics of inhibition (Figure 6C). Table 2 summarizes the determined and calculated kinetic and thermodynamic constants associated with inhibition of PGHS-2 by darbufelone.

In addition, Figure 8A shows the binding isotherm for PD0167570, an experimental PGHS inhibitor that is structurally similar to darbufelone (see Figure 1) but which is a very weak PGHS-2 inhibitor ($IC_{50} = 15$ μM with a 15-min preincubation, unpublished data). PD0167570 has an absorption spectrum similar to that of darbufelone (not shown). The binding isotherm for PD0167570 indicates that this compound binds PGHS-2 with $K_d = 0.25 \pm 0.03$ μM (Figure 8A). In contrast, saturating levels of SC-58125 (Figure 1), a

compound that inhibits PGHS-2 with $IC_{50} = 0.10$ μM (*17*), have no effect on the fluorescence of PGHS-2 (Figure 8A). This result is not altogether unexpected since the absorption spectrum of SC-58125 has no significant absorption at wavelengths above 300 nm (data not shown) and there is no significant spectral overlap to allow SC-58125 to quench PGHS-2 fluorescence. However, this observation indicates that SC-58125 and darbufelone bind to independent, distinct sites on PGHS-2.

Competitive Binding Experiments. PGHS-2 was titrated with darbufelone in the presence of potential competitor ligands. In the presence of varying levels (0.1−4 μM) of the selective PGHS-2 inhibitor SC-58125, the apparent K_d of darbufelone is unchanged (Figure 8B). Saturating levels (0.1−4 μM) of the related PGHS-2 inhibitor celecoxib (*17*) also have no effect on the affinity of darbufelone (not shown). In a control experiment, PGHS-2 was titrated with darbufelone in the presence of PD0167570. In these titrations, the apparent K_d for darbufelone increases linearly with increasing PD0167570 concentration (Figure 8B). Furthermore, the K_d for PD0167570 derived from this experiment (i.e., the *x*-intercept of Figure 8B) is 0.23 μM, in agreement with that determined for this inhibitor in the fluorescence quenching titrations with PGHS-2 alone ($K_d = 0.25$ μM, Figure 8A).

In the crystal structure determination of human PGHS-2 with R-57067, this inhibitor was bound deep in the cyclooxygenase active site (*40*). When the affinity of PGHS-2 for R-57067 was assessed later by fluorescence quenching methods, the quenched PGHS-2 fluorescence caused by 1.1 μM R-57067 was completely reversed by adding 50 μM arachidonic acid (*31*). R-57067 and arachidonate apparently cannot bind simultaneously to PGHS-2, suggesting that these ligands occupy the same binding site. To probe darbufelone's binding site, effects on darbufelone binding of compounds that are known to bind in the cyclooxygenase active site were assessed. ApoPGHS-2 alone or in the presence of 5 μM arachidonate, a ligand that has very minor effects on the enzyme fluorescence (*30*), binds darbufelone with high affinity ($K_d = 0.73$ and 0.80 μM, respectively). Likewise, holoPGHS-2 alone or in the presence of 40 μM arachidonate binds tightly to darbufelone ($K_d = 0.98$ and 0.91 μM, respectively). These results contrast with the reported effect of substrate on the binding of R-57067 (*31*). Darbufelone also binds equally well to PGHS-2 ($K_d = 1.17$ μM) following acetylation of the enzyme by acetyl salicylate (*30*). In addition, when PGHS-2 is titrated with darbufelone in the presence of 10 μM ibuprofen, a compound that inhibits PGHS-2 with a reported $K_i = 0.2$ μM but which has little effect on the fluorescence of PGHS-2 (*30*), the K_d of darbufelone is 0.97 μM.

DISCUSSION

Darbufelone was discovered as a dual inhibitor of cellular PG and LT biosynthesis (*24*, *25*). This di-*tert*-butyl phenol is orally active in animal models of inflammation (*25*) and is nonulcerogenic at very high doses (unpublished data). However, data from initial in vitro tests with purified PGHS-2 enzyme that was isolated using buffers containing Tween 20 detergent indicated that darbufelone was a very poor PGHS-2 inhibitor (*41*). The nonulcerogenic pharmacological nature of darbufelone, however, suggested that it might act at least in part as a PGHS-2 inhibitor in vivo. Since no mechanistic PGHS inhibition data have been reported for the di-*tert*-butyl phenols, we thought it would be informative to carry out such studies with darbufelone.

We have assessed inhibition with darbufelone of purified recombinant human PGHS enzymes that were isolated using buffers containing octylglucoside. In cyclooxygenase assays initiated after a 15-min preincubation with enzyme, darbufelone potently inhibits PGHS-2 but is much less effective with PGHS-1. This PGHS-2 selectivity is consistent with the nonulcerogenic nature of darbufelone. We observed a surprising adverse effect of Tween 20 on the in vitro potency of darbufelone. Darbufelone is a very weak inhibitor of PGHS-2 that was solubilized with, purified in, and assayed in buffers containing Tween 20. These assays contain 0.0001% (0.8 μM) Tween 20, a level much lower than the reported critical micellar concentration of 59 μM for this detergent (*42*). In light of the potent inhibition of cellular $PGF_{2\alpha}$ production that was observed previously with darbufelone (*25*), its lack of potency in assays with purified PGHS-2 (*41*) was unexpected. This ineffectiveness of darbufelone in assays containing Tween 20 correlates with a time-dependent, detergent-induced alteration in the absorption spectrum of darbufelone. This spectral change, however, does not occur in the presence of 0.0005% (17 μM) octylglucoside, a detergent with a reported critical micellar concentration of 22 mM (*43*). It is possible that Tween 20 (and perhaps other detergents as well) may similarly decrease the potency of other PGHS inhibitors. For example, in PGHS-2 inhibition assays carried out in the presence of 0.0001% Tween 20, we found the inhibitors indomethacin, SC-58125, and NS-398 were 10- to 100-fold less potent than when tested in the presence of 0.0005% octylglucoside (unpublished results). We emphasize that the buffers used with PGHS-2 in all of the mechanistic studies reported here contained octylglucoside, not Tween 20. Further, during the three-column PGHS-1 purification, the initially Tween 20-solubilized enzyme was bound sequentially to two different anion-exchange resins and the columns were washed exhaustively with buffer containing octylglucoside but no Tween 20. Thus, we believe our final PGHS-1 preparation, and the inhibition assays, likewise, contain no Tween 20.

We speculate that Tween 20 may lower the apparent critical micellar concentration of hydrophobic inhibitors, causing them to interact with other inhibitor molecules rather than with PGHS or may induce inhibitor molecules to aggregate together in a type of chain reaction mechanism. The absorption spectrum of Tween 20-treated darbufelone is well defined and does not have a high absorption component in the ultraviolet region (200−300 nm) as is characteristic of turbidity. Thus, we do not believe Tween 20 causes darbufelone to simply precipitate out of solution. In the small molecule crystal structure of darbufelone, its thiazolidinone and phenyl rings are twisted out-of-plane relative to each other (unpublished data). Tween 20 may bind to a darbufelone molecule and force its ring systems to be coplanar. The electron density in a planar darbufelone molecule would be delocalized throughout both the thiazolidinone and phenyl rings and the absorption spectrum of such a planar inhibitor would be of lower energy. Indeed, the Tween 20-induced spectral change in darbufelone shifts

the maximum absorption band from 360 nm (higher energy) to 405 nm (lower energy), as might be expected from such electron delocalization. Once planar, the Tween 20-bound darbufelone molecule could bind to and stack with a second darbufelone molecule and induce it to become coplanar. This type of chain reaction could continue until all of the darbufelone molecules were involved in an aggregation complex that was initiated by the Tween 20 molecule. In this type of chain reaction mechanism, one Tween 20 molecule could induce the aggregation of several darbufelone molecules. Although this chain reaction mechanism is consistent with the data we have in hand, we emphasize that at present it remains hypothetical.

Other PGHS inhibitors that are highly aromatic or nearly planar might be similarly affected by the presence of detergents. Because the adverse interaction occurs between the inhibitor and the detergent, one would expect such interference not only with PGHS-2 but also with PGHS-1 preparations that contain Tween 20. Thus, we caution that in studies of the inhibition of PGHS the use of buffers that contain Tween 20 may artificially mask the potency of certain cyclooxygenase inhibitors. In our experience, octylglucoside is an effective PGHS-2 solubilizing detergent that, at the levels used in our experiments, does not impair the mechanistic, kinetic, and binding studies with darbufelone.

Darbufelone is a time-dependent, slow-binding PGHS-2 inhibitor. With no preincubation, darbufelone is a very weak PGHS-2 inhibitor. After a 15-min preincubation, however, darbufelone is much more potent. In our inhibition kinetics studies, darbufelone was further found to be a slow-binding PGHS-2 inhibitor. The slow-binding inhibition of PGHS-2 by darbufelone is thus expressed in the kinetics of the second step of the binding interaction (i.e., in the slow $E{\cdot}I \rightleftarrows E^{*}{\cdot}I$ equilibrium). Although we find that darbufelone is very weak PGHS-1 inhibitor, further mechanistic studies of the kinetics of inhibition of this isoform are warranted.

Darbufelone quenches PGHS-2 fluorescence with high affinity. In fact, the K_d obtained in such titrations is similar to the K_i^* values we determined or calculated using various methods or rate constants (Table 2). Overall, the K_d data from fluorescence titrations, IC_{50} estimates at equilibrium, and K_i and K_i^* values from inhibition kinetics experiments all provide similar estimates of the initial and equilibrium affinity of PGHS-2 for darbufelone. Further, the relative magnitude of the rate constants k_5 and k_6 (i.e., $k_5 \gg k_6 > 0$) are consistent with a two-step, reversible slow-binding mechanism (*22*, *23*) of inhibition of PGHS-2 with darbufelone.

In the absence of crystal structure data, the location of the darbufelone binding site on PGHS-2 is unknown. In steady state kinetics experiments, we find that the inhibition of PGHS-2 by darbufelone is noncompetitive with arachidonic acid. Further, the potency of darbufelone is identical in the radiometric assays that do not contain TMPD as well as in the TMPD-linked spectrophotometric assays. Darbufelone does not interfere with the ability of TMPD to accurately report the cyclooxygenase activity. This suggests that darbufelone does not compete with TMPD as a cosubstrate for the peroxidase activity. Further, we have not observed any stimulation of PGHS-2 cyclooxygenase activity by darbufelone (or any other di-*tert*-butyl phenol tested). In fact, low concentrations of PGHS inhibitors such as ibuprofen, SC-58125, and others that contain no redox-active functional groups have been shown to stimulate PGHS-1 (*31*). Thus, stimulation of PGHS-1 activity does not necessarily require any involvement of the inhibitor with the peroxidase activity of PGHS-1. The finding that darbufelone binds with equal affinity to both apoPGHS-2 and heme-bound holoPGHS-2 suggests that this inhibitor does not interact with the heme cofactor to cause inhibition.

Compounds that are known to bind deep in the cyclooxygenase active site (i.e., arachidonate, acetyl salicylate, ibuprofen, SC-58125, and celecoxib) do not alter the affinity of darbufelone. These data, along with the finding that darbufelone is a noncompetitive inhibitor of PGHS-2, are consistent with the possibility that darbufelone may bind to a novel region of the protein, perhaps in the "lobby" of the enzyme (*44*). The lobby is located near the entrance to the arachidonic acid binding channel and near the membrane binding helices of PGHS. It has been suggested that the initial binding interactions of inhibitors with PGHS occur in this lobby (*44*). With time-dependent PGHS-2 inhibitors, the $E{\cdot}I$ complex then undergoes a conformational transition such that the inhibitor becomes more tightly bound. For many selective PGHS-2 inhibitors, this transition may occur as the inhibitor moves from the lobby to its final binding site deep within the cyclooxygenase active-site channel. Indeed, the recent report of a modeling study has identified a putative second binding site for inhibitors such as SC-58125 and flurbiprofen in or near the lobby (*45*). Initial weak binding in the lobby with weak inhibitory activity is translated to potent inhibition by a time-dependent conformational change that is detected experimentally as slow-binding inhibition. Although PD0167570 competes strongly with darbufelone for binding to PGHS-2, other factors (i.e., the slow $E{\cdot}I \rightleftarrows E^{*}{\cdot}I$ conformational equilibrium) must play a role in overall inhibitory potency since PD0167570 is a very weak PGHS-2 inhibitor. The time-dependent inhibition of PGHS-2 with darbufelone (or other di-*tert*-butyl phenols) may be the result of utilization of a novel binding site with unique kinetic characteristics. Darbufelone may bind in the lobby of PGHS-2, which then undergoes a slow conformational change to yield tightly bound inhibitor still within the lobby and manifesting itself as a noncompetitive inhibitor. Although more detailed studies of the mechanism of noncompetitive inhibition of PGHS-2 by darbufelone will be necessary to determine whether darbufelone binds to free enzyme and the enzyme−substrate complex with similar or different affinities, the results presented here support a novel mode of inhibition of PGHS-2 by darbufelone by a slowly reversible, two-step mechanism.

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