Multiple Inhibitor Analysis of the Brequinar and Leflunomide Binding Sites on Human Dihydroorotate Dehydrogenase†

Jeremy E. McLean,‡ Edie A. Neidhardt,§,|| Trudy H. Grossman,§,⊥ and Lizbeth Hedstrom*

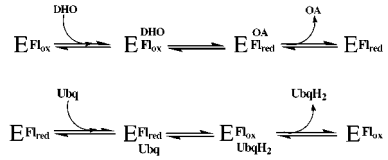
Program in Biophysics and Structural Biology, Brandeis University, 415 South Street, Waltham, Massachusetts 02454, Procept Inc., 840 Memorial Drive, Cambridge, Massachusetts 02139, and Department of Biochemistry, Brandeis University, 415 South Street, Waltham, Massachusetts 02454

Received August 1, 2000; Revised Manuscript Received November 16, 2000

ABSTRACT: Brequinar and the active metabolite of leflunomide, A77 1726, have been clearly shown to inhibit human dihydroorotate dehydrogenase (DHODH), but conflicting mechanisms for their inhibition have been reported. DHODH catalyses the conversion of dihydroorotate (DHO) to orotate concurrent with the reduction of ubiquinone. This study presents data that indicates brequinar is a competitive inhibitor versus ubiquinone; A77 1726 is noncompetitive versus ubiquinone and both are uncompetitive versus DHO. 2-Phenyl 5-quinolinecarboxylic acid (PQC), the core moiety of brequinar also shows competitive inhibition versus ubiquinone. Multiple inhibition experiments indicate that PQC (and thus brequinar) and A77 1726 have overlapping binding sites. Both PQC and A77 1726 are also mutually exclusive with barbituric acid (a competitive inhibitor versus DHO). In addition, we failed to observe brequinar binding to E·orotate by isothermal titration calorimetry (ITC). These results indicate that the E·DHO·inhibitor and E·orotate·inhibitor ternary complexes do not form. The absence of these complexes is consistent with the two-site ping-pong mechanism reported for DHODH. This kinetic data suggests that recent crystal structures of human DHODH complexed with orotate and A77 1726 or brequinar may not represent the relevant physiological binding sites for these inhibitors [Liu, S., Neidhardt, E. A., Grossman, T. H., Ocain, T., and Clardy J. (2000) *Structure* 8, 25−33].

Dihydroorotate dehydrogenase (DHODH;[1] EC 1.3.3.1) is the fourth enzyme in the de novo pyrimidine nucleotide synthesis pathway. It catalyses the only oxidation/reduction reaction in that pathway: converting DHO to orotate with the aid of a flavin cofactor and an electron acceptor. The cosubstrate electron acceptor used by DHODH varies in different organisms. In the human enzyme, the flavin cofactor is FMN and ubiquinone is the second substrate. DHODH from higher eukaryotes exhibits a two-site ping-pong mechanism as depicted in Scheme 1 (*1*, *2*). In the first site, DHO is oxidized to orotate, reducing the FMN cofactor. Orotate is then released before ubiquinone binds in a second site and reoxidizes the cofactor. While DHO and orotate are soluble, ubiquinone, with its long isoprenoid tail (Figure 1), is confined to membranes. Thus, DHODH must also be membrane associated to utilize ubiquinone. The human and bovine enzymes are associated with the mitochondrial inner membrane (*3*), where the reduced ubiquinone product can be reoxidized at complex III of the electron respiratory chain.

Scheme 1



Several anti-tumor and immunosuppressive drugs target human DHODH (*4*, *5*). The two most promising drugs (see Figure 1) are brequinar (antitumor and immunosuppressive) and leflunomide (immunosuppressive). The latter has been FDA-approved for use in rheumatoid arthritis under the trade name of Arava (Hoechst Marion Roussel). A ring-opening reaction converts leflunomide into its active metabolite, A77 1726 (Figure 1). Although both brequinar and A77 1726 clearly inhibit human DHODH, conflicting mechanisms of inhibition have been reported. Brequinar resembles ubiquinone (Figure 1) and would therefore be expected to bind in the ubiquinone active site. In a two-site ping-pong mechanism such a ubiquinone analogue will be an uncompetitive inhibitor versus DHO and a competitive inhibitor versus ubiquinone. However, most studies report that brequinar is a noncompetitive inhibitor versus both ubiquinone and DHO (*6*, *7*). In contrast atovaquone (Figure 1), which is very similar to brequinar, does inhibit competitively versus ubiquinone and uncompetitively versus DHO (*8*). The mechanism of A77 1726 inhibition is also controversial: inhibition versus DHO has been reported as either uncompetitive or noncompetitive (*9*−*11*). A77 1726 is reported as

† This work was funded by NIH GM54403 and a grant from the Markey Charitable Trust to Brandeis University. J.E.M. is a Howard Hughes Medical Institute predoctoral fellow.

* To whom correspondence should be addressed. Department of Biochemistry, Brandeis University. Phone: (781) 736-2333. E- mail: hedstrom@brandeis.edu.

‡ Program in Biophysics and Structural Biology, Brandeis University.

§ Procept Inc.

|| Present address: Genetics Institute, Andover, MA.

⊥ Present address: Vertex Pharmaceuticals, 130 Waverly St., Cambridge, MA 02139.

[1] Abbreviations: DHODH, dihydroorotate dehydrogenase; FMN, flavin mononucleotide; DHO, dihydroorotate; CoQ_0, Coenzyme Q_0; PQC, 2-phenyl 5-quinolinecarboxylic acid; DCIP, 2,6-dichlorophenolindophenol; Ubq, ubiquinone; HNBB, 2-hydroxy-5-nitrobenzyl bromide; barb, barbituric acid; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry.

10.1021/bi001810q CCC: $20.00
Published on Web 01/25/2001

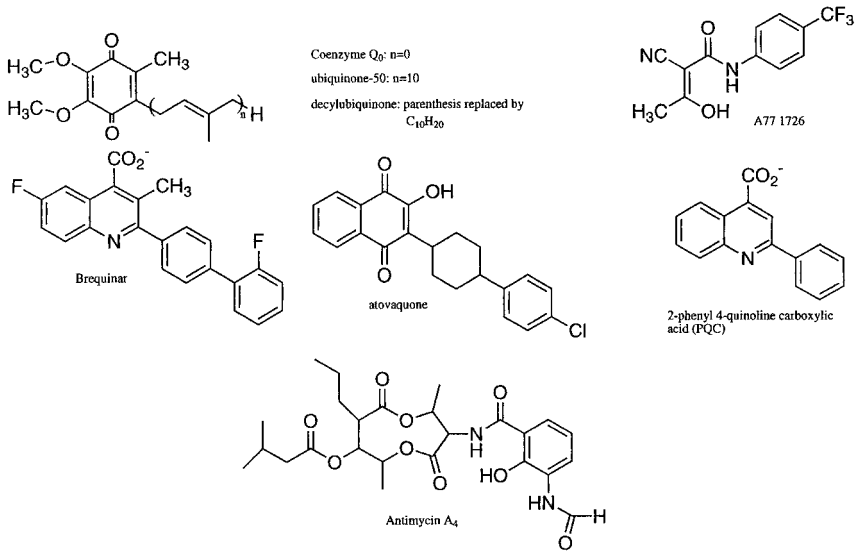


FIGURE 1: DHODH substrate and inhibitors.

either a competitive or a noncompetitive inhibitor versus ubiquinone (*9*−*11*).

We have reexamined the mechanism of brequinar and A77 1726 inhibition of human DHODH utilizing a different assay (*12*). Both these drugs are uncompetitive inhibitors versus DHO. Brequinar is a competitive inhibitor versus ubiquinone but A77 1726 is noncompetitive versus ubiquinone. Multiple inhibition experiments suggest that the binding sites of brequinar and A77 1726 overlap. Furthermore both multiple inhibition and isothermal titration calorimetry (ITC) experiments suggest that brequinar and A77 1726 do not bind to the E·orotate complex. This result suggests that recently published E·orotate·inhibitor structures do not represent high affinity complexes (*13*).

MATERIALS AND METHODS

Materials. DHO, CoQ_0, orotate, 2-phenyl 4-quinolinecarboxylic acid (PQC), decylubiquinone and antimycin A_4 were purchased from Sigma Chemical Co. (St. Louis, MO). Barbituric acid was obtained from Eastman Kodak Co. (Rochester, NY). A77 1726 and brequinar were acquired from Procept Inc. Human his_{10}-tagged DHODH was purified as previously described (*2*).

Enzyme Inhibition. All DHODH assays contained 150 mM KCl and 50 mM Tris (pH 8.0). The concentrations of substrates CoQ_0 (45−360 μM) and DHO (5−100 μM) as well as those of the inhibitors brequinar (1−20 nM), A77 1726 (25−500 nM), PQC (8−200 μM), and antimycin A_4 (3−60 μM) were varied appropriately for each experiment. CoQ_0 was used as the ubiquinone substrate since it is soluble in water and thus eliminates the need for Triton X-100. When decylubiquinone (1−20 μM) was used as a substrate, 0.1% Triton X-100 was added to the assay buffer. The production of orotic acid was monitored spectrophotometrically at 287 nm for CoQ_0 (ϵ = 6040 M^{-1} cm^{-1}) and 296 nm for decylubiquinone (ϵ = 4300 M^{-1} cm^{-1}) at 25 °C using a Hitachi U-2000 spectrophotometer as per Hines, Keys, and Johnston (1986). Initial velocity data for A77 1726, PQC and antimycin A_4 inhibition was fit to competitive, uncompetitive and noncompetitive inhibition equations (eqs 1, 2, and 3, respectively) using KinetAsyst software (Intellikinetics). However, the brequinar inhibition was in tight-binding range so could not be fit with these generalized equations. Instead the Ackermann tight-binding equations (*14*) for competitive (eq 4) and uncompetitive (eq 5) inhibition were used to fit the data in the Sigmaplot software (Jandel Scientific). Multiple inhibition experiments were fit with the Sigmaplot software (eq 6)

$$v = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A} \quad (1)$$

$$v = \frac{VA}{K_a + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (2)$$

$$v = \frac{VA}{K_a\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (3)$$

$$v = \frac{VA}{2E(K_a + A)}\left[E - K_i\left(1 + \frac{A}{K_a}\right) - I + \sqrt{\left[K_i\left(1 + \frac{A}{K_a}\right) + I + E\right]^2 - 4IE}\right] \quad (4)$$

$$v = \frac{VA}{2E(K_a + A)}\left[E - K_i\left(1 + \frac{K_a}{A}\right) - I + \sqrt{\left(K_i\left(1 + \frac{A}{K_a}\right) + I + E\right)^2 - 4IE}\right] \quad (5)$$

$$v = \frac{v_o}{1 + \frac{I}{k_1} + \frac{J}{k_2} + \frac{IJ}{\alpha k_1 k_2}} \quad (6)$$

where v is initial velocity, v_o is initial velocity in the absence of inhibitor, V is maximum velocity, A is substrate concentration, K_a is the apparent Michaelis constant for substrate, K_{ii} and K_{is} are intercept and slope inhibition constants respectively, I and J are the concentrations of the inhibitors,

E is the concentration of the enzyme and K_i is an apparent inhibition constant. In the multiple inhibition experiments, K_1 and K_2 are the apparent inhibition constants for I and J, respectively; α is the interaction constant between these two inhibitors.

Pre-steady-state studies of the DHO half-reaction were performed on an Applied Photophysics Sx.17MV stopped flow spectrophotometer. The reduction of the flavin cofactor was monitored at 450 nm ($\Delta\epsilon = 1.22 \times 10^4$ M^{-1} cm^{-1}) at 25 °C.

Isothermal Titration Calorimetry. The titrations of brequinar into orotate-bound DHODH were performed on a MicroCal VP−ITC titration calorimeter. Brequinar (250 μM) was titrated into 1.4 mL of 3−6 μM DHODH using 2 μL injections. Enzyme was dialyzed into the appropriate buffer containing orotate. Brequinar was dissolved directly into the final dialysate. Temperature was either 26 or 35 °C. Buffers used were either 50 mM Tris pH 8.0, 150 mM KCl and 260 μM orotate or 50 mM phosphate buffer, pH 7.9, 150 mM KCl and 260 μM orotate.

Tryptophan Modification. DHODH (50 nM) in 0.1 M sodium acetate (pH 5.5) was inactivated with 2.4 mM 2-hydroxy-5-nitrobenzylbromide with or without brequinar (55 nM) or A77 1726 (10 μM). The activity of the inactivated protein was measured after dialysis into 150 mM KCl and 50 mM Tris, pH 8.0 at 0 °C.

RESULTS

Orotate Production Assay. The most commonly used assay for measuring DHODH activity is a coupled assay using 2,6-dichlorophenol-indophenol (DCIP) (*15*, *16*). In this assay, DCIP reoxidizes the ubiquinol produced by DHODH. Reduction of DCIP causes a decrease in absorbance at 600 nm that can be easily monitored. However, DCIP is also a substrate for DHODH (*12*). Under typical assay conditions, 10−30% of the observed activity (depending on the quinone substrate) is due to the direct reduction of DCIP by DHODH (*12*). The presence of two substrates complicates an inhibition experiment and will lead to errors if both substrates are not explicitly considered in analysis. Therefore, we utilized an assay monitoring the production of orotate at 287 nm, the isobestic point for the oxidized and reduced forms of CoQ_0 (*12*). CoQ_0 is a soluble form of ubiquinone that lacks the isoprenoid tail of natural substrates (Figure 1).

Antimycin A_4 Inhibition. The respiratory toxin, antimycin A_4 (structure in Figure 1) specifically inhibits cytochrome bc_1 at a site of ubiquinone reduction (*17*−*19*). As antimycin A_4 targets ubiquinone reduction, it could possibly also inhibit DHODH. Antimycin A_4 is indeed an inhibitor of human DHODH: an uncompetitive inhibitor versus DHO (K_{ii} = 72 μM) and a competitive inhibitor versus CoQ_0 (K_{is} = 140 μM; Table 1). Antimycin A_4 is much less potent with DHODH than with cytochrome bc_1 [$K_d = 3.2 \times 10^{-11}$ M (*20*)]. Despite this lowered potency, antimycin A_4 acts as a ubiquinone analogue. Further the inhibition pattern is consistent with the two-site ping-pong mechanism previously proposed (*1*, *2*).

Brequinar Inhibition. In our experiments the concentrations of DHODH and brequinar are comparable so tight binding analysis is required. Brequinar is an uncompetitive inhibitor versus DHO (K_i = 3.2 nM; eq 5; Table 1) unlike the previous reports of noncompetitive inhibition (*6*, *7*).

Table 1: Kinetic Inhibition Data[a]

inhibitor	substrate	mode	K_{is} (μM)	K_{ii} (μM)
Brequinar	DHO	uc		$(3 \pm 2) \times 10^{-3}$
	CoQ_0	c	$(0.45 \pm 0.06) \times 10^{-3}$	
PQC	DHO	uc		6.7 ± 0.4
	CoQ_0	c	5.3 ± 0.5	
A77 1726	DHO	uc		$(18 \pm 1.4) \times 10^{-3}$
	CoQ_0	nc	$(36 \pm 19) \times 10^{-3}$	$(34 \pm 13) \times 10^{-3}$
	Decylubiquinone	nc	$(137 \pm 74) \times 10^{-3}$	$(259 \pm 84) \times 10^{-3}$
Antimycin A_4	DHO	uc		72 ± 10
	CoQ_0	nc	140 ± 17	

[a] 150 mM KCl, 50 mM Tris, pH 8.0, 25 °C. When DHO is varied, [CoQ_0] is 100 μM; when CoQ_0 or decylubiquinone are varied, [DHO] is 25 μM.

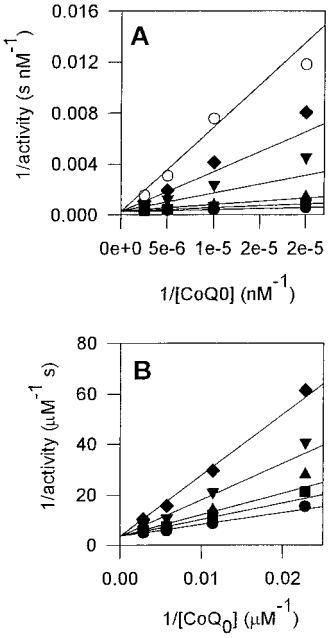


FIGURE 2: Inhibition of human DHODH by brequinar and PQC. (A) Brequinar inhibition with CoQ_0 as the varied substrate. The concentrations of brequinar were (●) 0, (■) 1.01 nM, (▲) 2.02 nM, (▼) 5.04 nM, (◆) 10.1 nM, and (○) 20.2 nM. The lines are the best fit to the Ackerman competitive inhibition (eq 4 in the Materials and Methods). (B) PQC inhibition with CoQ_0 as the varied substrate. PQC concentrations are (●) 0, (■) 2.24 μM, (▲) 4.48 μM, (▼) 11.2 μM, and (◆) 22.4 μM. Lines represent the best fit to a competitive inhibition (eq 1 in the Materials and Methods).

In previous inhibition experiments where brequinar inhibition of DHODH was monitored using the DCIP assay, the inhibition was noncompetitive versus ubiquinone substrates [rat enzyme (*8*)]. Using the DCIP assay, we also observe noncompetitive inhibition versus CoQ_0 (data not shown). However, brequinar is competitive versus CoQ_0 in the orotate production assay (Figure 2; K_i = 0.45 nM). This value is consistent with a reported IC_{50} of 0.46 nM for human enzyme determined by following orotate production with solubilized human mitochondrial membranes (containing ubiquinone-50; Figure 1) using [^{14}C]DHO (*10*). However our reported K_i is lower than that from other reports (*7*). This difference can probably be attributed to the DCIP assay. Thus brequinar also exhibits inhibition consistent with that of a ubiquinone analogue.

PQC Inhibition. 2-Phenyl 4-quinoline carboxylic acid (PQC), the core moiety of brequinar (Figure 1), is a

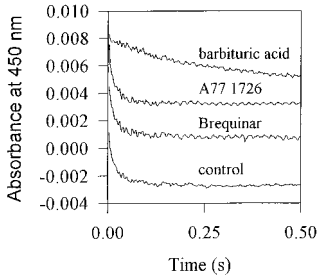


FIGURE 3: Inhibition of the pre-steady-state DHO half reaction. Each reaction trace is the average of at least nine individual reactions with 10 μM DHO. The reactions were performed in 150 mM KCl and 50 mM Tris, pH 8.0 at 25 °C with a final enzyme concentration of 200 nM. The final concentrations of inhibitor were brequinar, 50 nM; A77 1726, 7.1 μM; and barbituric acid, 420 μM.

competitive inhibitor versus CoQ_0 (Figure 2). The value of K_i for PQC (K_{is} = 5.3 μM; from eq 1) is at least 10^4-fold higher than that of brequinar. PQC is an uncompetitive inhibitor versus DHO (K_{ii} = 6.7 μM). Thus PQC is also a ubiquinone analogue.

A77 1726 Inhibition. A77 1726 is an uncompetitive inhibitor versus DHO (K_{ii} = 18 nM) and a noncompetitive inhibitor versus CoQ_0 (K_{is} = 36 nM, K_{ii} = 34 nM; see Table 1). However, the values of K_i are 1−2 orders of magnitude lower than the literature values for human DHODH [K_{ii} = 0.94 μM versus DHO; K_{is} = 1.1 μM and K_{ii} = 1.0 μM versus decylubiquinone: see Figure 1 (*9*)]. Only part of this difference between the values of K_i (~10-fold) can be attributed to the presence of DCIP in the previous experiments. To determine if the remaining difference results from the use of different quinone substrates, we repeated the inhibition experiments using decylubiquinone. A77 1726 remained a noncompetitive inhibitor with decylubiquinone but the values of K_i were ~8-fold higher than those with CoQ_0 (K_{is} = 0.13 μM; K_{ii} = 0.25 μM). Brequinar, PQC and barbituric acid show no change in the value of K_i when decylubiquinone is the substrate (data not shown). Thus, the value of K_i for only A77 1726 depends on the quinone substrate. The sole difference between CoQ_0 and decylubiquinone is the presence of an alkyl chain in decylubiquinone which mimics the isoprenoid tail of the natural substrate (Figure 1). The quinone dependence of A77 1726 inhibition suggests that this inhibitor binds near the hydrophobic tail of ubiquinone.

Inhibition of the DHO Half-Reaction. Since DHODH operates by a ping-pong mechanism, the DHO half-reaction can be monitored independently by observing the reduction of FMN at 450 nm. Figure 3 shows the reaction in the presence and absence of saturating concentrations (~100 × K_i) of brequinar (50 nM) or A77 1726 (7.1 μM). Neither brequinar nor A77 1726 significantly affect the progress curve: all these reactions are complete within 30 ms. In contrast the reaction which contains barbituric acid (420 μM; $K_i \approx$ 200 μM), a competitive inhibitor versus DHO (*1*, *2*), shows appreciably slower kinetics: the reaction is not complete within 150 ms. The reaction is complex: the traces could not be fit to either single or double exponential equations. Unfortunately, we are unable to monitor the CoQ_0 half-reaction by this method due to an inability to maintain anaerobic conditions and the overlapping UV−vis spectra of flavin and ubiquinone.

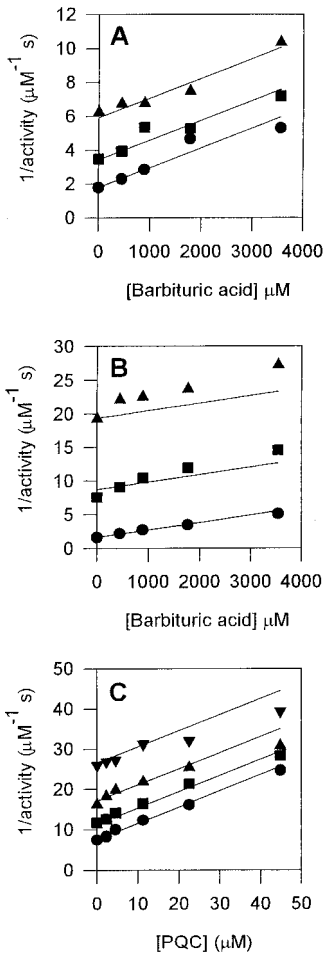


FIGURE 4: Multiple inhibition of DHODH. Final concentrations of DHO and CoQ_0 for all experiments were 10.2 and 94 μM, respectively, buffer conditions were 150 mM KCl and 50 mM Tris, pH 8.0, at 25 °C. All graphs show the fit to the multiple inhibition equation with $\alpha = \infty$ (eq 6 from Materials and Methods). (A) Multiple inhibition with PQC and barbituric acid. PQC concentrations were (●) 0, (■) 8.82 μM, and (▲) 22.0 μM. The fits give a K_i for PQC of 9.9 μM and for barbituric acid of 1.5 mM. (B) Multiple inhibition with A77 1726 and barbituric acid. A77 1726 concentrations were (●) 0, (■) 214 nM, and (▲) 537 nM. The fits give a K_i of 49 nM for A77 1726 and of 1.5 mM for barbituric acid. (C) Multiple inhibition with A77 1726 and PQC. A77 1726 concentrations were (●) 0, (■) 21.4 nM, and (▲) 53.7 nM. The fits give a K_i of 43 nM for A77 1726 and 19 μM for PQC.

Multiple Inhibition. Multiple inhibitor experiments probe how two inhibitors interact. This interaction can be used to infer the relative positioning of the inhibitor binding sites. We used multiple inhibition experiments to determine the relative positioning of the ubiquinone, DHO and A77 1726 binding sites. This is possible as barbituric acid and PQC (as competitive inhibitors) will bind in the DHO and ubiquinone sites, respectively (unfortunately, tight-binding inhibitors such as brequinar cannot be used in multiple inhibition experiments). The plots for all combinations of A77 1726, PQC, and barbituric acid show a pattern of parallel lines (Figure 4), which indicate that the interaction constant $\alpha = \infty$ (refer to eq 6). These results indicate that all of the inhibitors are mutually exclusive.

Isothermal Titration Calorimetry. Titration calorimetry experiments measure the heat evolved in the binding of ligands to protein. We analyzed the binding of brequinar to DHODH·orotate by titrating brequinar into DHODH in the

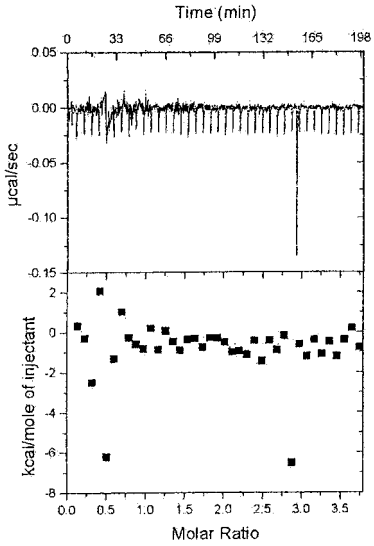


FIGURE 5: Isothermal titration calorimetry of brequinar titrated into DHODH and orotate. (Top) Data from the titration of 250 μM brequinar (2 μL aliquots) into 1.4 mL of 4.9 μM DHODH at 26 °C. Both inhibitor and protein are in 50 mM Tris, pH 8.0, 150 mM KCl, and 230 μM orotate. (Bottom) Buffer corrected integrated enthalpies for each injection. The integrated enthalpies of a titration of brequinar into buffer alone were subtracted from the integrated enthalpies of the data from the top panel.

Table 2: Tryptophan Modification by HNBB[a]

experimental conditions	activity (μM s^{-1})
unmodified control	0.12
+ HNBB (2.4 mM)	0.0062
+ HNBB and DHO (100 μM)	0.014
+ HNBB, DHO and Brequinar (1.9 μM)	0.0039
+ HNBB, DHO and A77 1726 (4.0 μM)	0.0088

[a] Modified in 90 mM sodium acetate, pH 5.4, overnight at 4 °C. Activity monitored in 150 mM KCl and 50 mM Tris, pH 8.0, at 25 °C with 25 μM DHO and 100 μM CoQ_0.

presence of saturating orotate. Formation of the DHODH·orotate complex was verified by the observation of the characteristic red-shift of the FMN spectra from 450 to 475 nm (*21*). The brequinar titration of DHODH·orotate in Tris buffer at 26 °C only shows the heat of dilution (Figure 5). Titrations were repeated at 35 °C and in phosphate buffer to ensure that the binding was not masked by entropic or buffer effects (data not shown). These results suggest that brequinar does not bind DHODH·orotate. Under the conditions of these experiments, a conservative lower limit for the K_d of brequinar binding to DHODH·orotate would be 5 μM.

Tryptophan Modification. Human DHODH contains a single tryptophan residue (Trp362). This tryptophan exhibits weak fluorescence resonance energy transfer with the FMN moiety (data not shown). This energy transfer suggests that Trp362 may be within 30 Å of the FMN cofactor. Modification of Trp362 by HNBB inactivates DHODH (see Table 2). However, DHO, brequinar, and A77 1726 do not protect the enzyme from inactivation.

DISCUSSION

DHODH is an important therapeutic target and the elucidation of the mechanism of DHODH inhibitors is critical to the further development of DHODH-based therapies. Brequinar resembles ubiquinone and the ubiquinone analogue, atovoquone (Figure 1). As such, brequinar should exhibit competitive inhibition versus ubiquinone. However previous investigations report that brequinar is a noncompetitive inhibitor versus ubiquinone substrates (*6*, *8*). Our results suggest that this discrepancy can be attributed to the use of the DCIP assay. With an orotate production assay, brequinar is a competitive inhibitor versus CoQ_0. A core moiety of brequinar, PQC, is also a competitive inhibitor versus CoQ_0, although with a K_i 10^4-fold higher than brequinar. PQC lacks the last phenyl ring of brequinar (Figure 1) which previous work has indicated contributes 3 orders of magnitude to the binding constant (*22*).

As ubiquinone analogues, brequinar and PQC should be uncompetitive inhibitors versus DHO, due to the two-site ping-pong enzyme mechanism. Our results do show uncompetitive inhibition versus DHO for these inhibitors. In addition, the pre-steady-state experiments examining the DHO half-reaction also support this outcome: brequinar does not affect DHO-mediated flavin reduction. These pre-steady-state results also suggest that brequinar and PQC do not bind the oxidized flavin form of DHODH ($E{\cdot}Fl_{ox}$; see Scheme 1).

The structure of A77 1726 (Figure 1) does not resemble that of ubiquinone. Thus A77 1726, unlike brequinar, may not be a ubiquinone analogue. Our results and several previous reports indicate that the inhibition by A77 1726 versus ubiquinone substrates is noncompetitive (*9*, *10*). In addition the inhibition of A77 1726 is dependent on the quinone substrate. Our results indicate that the value of K_i becomes higher with substrates that have a hydrophobic tail. These observations suggest that A77 1726 binds a site close to the isoprenoid tail of ubiquinone. A77 1726 is an uncompetitive inhibitor versus DHO (our results and ref *9*). A77 1726 also does not affect the DHO half-reaction which suggests that A77 1726 does not bind $E{\cdot}Fl_{ox}$.

The multiple inhibition experiments indicate that PQC (and, presumably also, brequinar), A77 1726 and barbituric acid are all mutually exclusive. The simplest explanation for mutually exclusive inhibition is overlapping binding sites. However, PQC and barbituric acid occupy the active sites for ubiquinone and DHO respectively which should be distinct. This contradiction can be resolved as the two site ping-pong mechanism precludes the formation of an E·DHO·Ubq ternary complex. Thus competitive inhibitors for these substrates should not bind concurrently and would be mutually exclusive: the E·barb·PQC, E·orotate·PQC, E·DHO·PQC, and E·barb·Ubq ternary complexes should not form. This result is confirmed by ITC experiments: brequinar does not bind E·orotate within the limits of detection which indicates that K_d must be greater than 5 μM. Thus E·orotate·brequinar would not form at the concentrations used in the steady-state assays, nor would this ternary complex form under therapeutic concentrations of brequinar.

The multiple inhibitor experiments indicate that A77 1726 and PQC (and presumably brequinar) are mutually exclusive. This result suggests that the binding sites of A77 1726 and PQC overlap. Initially, this conclusion appears to conflict with the observation that A77 1726 is a noncompetitive inhibitor versus CoQ_0. Noncompetitive inhibition indicates that the $E{\cdot}CoQ_0{\cdot}$A77 1726 complex forms. As a CoQ_0

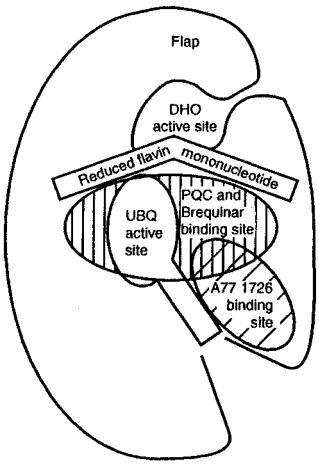


FIGURE 6: Proposed location of inhibitor binding sites on DHODH.

analogue, PQC might also be expected to form a ternary complex with enzyme and A77 1726. However PQC is much larger than CoQ_0 (see Figure 1). Thus PQC could extend beyond the quinone site into the A77 1726 site (see Figure 6). Like PQC, A77 1726 is also mutually exclusive with barbituric acid. This observation suggests that the A77 1726 binding site is also not accessible in the E·DHO or E·orotate complexes.

X-ray crystal structures of human DHODH complexed with orotate and either a brequinar analogue (missing the fluorine on the phenyl ring) or A77 1726 were recently determined (*13*). The protein is an α/β barrel with the FMN attached to one end of the barrel. Orotate stacks over the flavin and defines the DHO site. A flap traps the orotate within the active site. Brequinar and A77 1726 bind in a short tunnel that leads to the edge of the flavin, near the 7′-carbon. This tunnel could be the ubiquinone site.

These structures confirm several of the conclusions from our work. Brequinar and A77 1726 do have overlapping binding sites (as indicated by the multiple inhibition data). A77 1726 binds within the tunnel which would consequently be close to the hydrophobic tail of ubiquinone. In addition, the structures also reveal that Trp-362 is close enough to the flavin (17 Å) to explain the weak FRET effect. More importantly, this tryptophan is only 7.6 Å from brequinar bound in the tunnel. Furthermore the indole ring is directed into bulk solvent instead of into the tunnel so this tryptophan could be modified despite the presence of DHO, brequinar or A771726. As the tryptophan is so close to the tunnel, any change in conformation of that residue could affect the tunnel and presumably inhibit the reaction.

However, there are a few inconsistencies between the structures and our data. The binding of A77 1726 in (and blocking) the tunnel is not consistent with noncompetitive inhibition versus CoQ_0. There is no appropriate empty (or waterfilled) cavity close to the flavin in the structure to accommodate CoQ_0 as well as A77 1726 (formation of the E·Ubq·A77 1726 ternary complex is the simplest explanation for noncompetitive inhibition). The tunnel is reported to be less ordered in the A77 1726 structure than in the brequinar structure (*13*). Perhaps the tunnel retains sufficient plasticity in the presence of A77 1726 to allow ubiquinone substrates to bind.

However, more importantly, our results indicate that DHODH does not bind orotate concurrently with either A77 1726 or brequinar. Yet both structures are crystallized as E·orotate·inhibitor. Furthermore, while our data indicates that brequinar and A77 1726 do not impede flavin reduction and consequently should not bind to DHODH containing oxidized flavin, both structures seem to contain $E{\cdot}Fl_{ox}$. The inconsistencies between the structural and kinetic data are most probably due to the exceptionally high concentration of inhibitors used during crystallization (1 mM for both brequinar and A77 1726). These concentrations are 4−6 orders of magnitude above the K_i for A77 1726 (∼100 nM) and brequinar (∼1 nM) and 200-fold higher than the lower limit of K_d obtained from ITC. Thus the structures probably show A77 1726 and brequinar in low affinity binding modes. We would not be able to observe lower affinity binding modes in our experiments. Interestingly, neither inhibitor forms the perfectly complementary contacts with the enzyme generally observed with high affinity inhibitor complexes. Thus, these crystal structures of human DHODH complexed with A77 1726 and brequinar may not accurately represent the relevant physiological binding sites for these inhibitors.

ACKNOWLEDGMENT

We wish to thank Jon Clardy and Shenping Liu for the coordinates of the DHODH-inhibitor structures. Thanks also to Elizabeth Arkema for preliminary work on antimycin A_4 and to Gunther Kern and Stewart Fisher at Astrazeneca R&D Boston for help with ITC data collection. Cecilia Bastos and Zhan Shi synthesized the brequinar and A77 1726 used in this work.

REFERENCES

1. Hines, V., and Johnston, M. (1989) *Biochemistry 28*, 1222−1226.
2. Neidhardt, E. A., Punreddy, S. R., McLean, J. E., Hedstrom, L. K., and Grossman, T. H. (1999) *J. Mol. Microbiol. Biotechnol. 1*, 183−188.
3. Rawls, J., Knecht, W., Diekert, K., Lill, R., and Loffler, M. (2000) *Eur. J. Biochem. 267*, 2079−2087.
4. Williamson, R. A., Yea, C. M., Robson, P. A., Curnock, A. P., Gadher, S., Hambleton, A. B., Woodward, K., Bruneau, J. M., Hambleton, P., and Moss, D., et al. (1995) *J. Biol. Chem. 270*, 22467−22472.
5. Chen, S. F., Ruben, R. L., and Dexter, D. L. (1986) *Cancer Res. 46*, 5014−5019.
6. Chen, S. F., Perrella, F. W., Behrens, D. L., and Papp, L. M. (1992) *Cancer Res. 52*, 3521−3527.
7. Knecht, W., and Loffler, M. (1998) *Biochem. Pharmacol. 56*, 1259−1264.
8. Knecht, W., Henseling, J., and Loffler, M. (2000) *Chem. Biol. Interact. 124*, 61−76.
9. Knecht, W., Bergjohann, U., Gonski, S., Kirschbaum, B., and Loffler, M. (1996) *Eur. J. Biochem. 240*, 292−301.
10. Greene, S., Watanabe, K., Braatz-Trulson, J., and Lou, L. (1995) *Biochem. Pharmacol. 50*, 861−867.
11. Davis, J. P., Cain, G. A., Pitts, W. J., Magolda, R. L., and Copeland, R. A. (1996) *Biochemistry 35*, 1270−1273.
12. Hines, V., Keys, L. D. d., and Johnston, M. (1986) *J. Biol. Chem 261*, 11386−11392.
13. Liu, S., Neidhardt, E. A., Grossman, T. H., Ocain, T., and Clardy, J. (2000) *Struct. Folding Des. 8*, 25−33.
14. Ackermann, W. W., and Potter, V. R. (1949) *Proc. Soc. Exp. Biol. Med. 72*, 1−9.
15. Forman, H. J., and Kennedy, J. (1977) *Prep. Biochem. 7*, 345−355.

16. Miller, R. W. (1978) *Methods Enzymol. 51*, 63−69.
17. von Jagow, G., and Link, T. A. (1986) *Methods Enzymol. 126*, 253−271.
18. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) *Nature 392*, 677−684.
19. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) *Science 281*, 64−71.
20. Berden, J. A., and Slater, E. C. (1972) *Biochim. Biophys. Acta 256*, 199−215.
21. Bjornberg, O., Gruner, A. C., Roepstorff, P., and Jensen, K. F. (1999) *Biochemistry 38*, 2899−2908.
22. Chen, S. F., Papp, L. M., Ardecky, R. J., Rao, G. V., Hesson, D. P., Forbes, M., and Dexter, D. L. (1990) *Biochem. Pharmacol. 40*, 709−714.

BI001810Q