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DIFFERENTIAL SUSCEPTIBILITY OF DIHYDROOROTATE DEHYDROGENASE/OXIDASE TO BREQUINAR SODIUM (NSC 368 390) *IN VITRO*

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Abstract—To verify the assumption of a specific and potent drug action on *de novo* pyrimidine biosynthesis, isolated dihydroorotate dehydrogenase (DHO-DH) (EC 1.3.3.1) was exposed to Brequinar Sodium™ (6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt, NSC 368 390) (Brequinar). The membrane-bound DHO-DH was purified to apparent homogeneity (25,000-fold) from rat liver mitochondria in six steps via detergent extraction and subsequent chromatography using the dye ligand Matrex™ Gel Orange A. Using molecular mechanistic studies (MM2) this ligand was found to mimic closely the stereochemical conformation of Brequinar. SDS–PAGE revealed two protein bands for the purified enzyme with apparent molecular masses of 58 (major) and 68 kDa (minor). *In vitro*, two modes of action of the DHO-DH are possible: (i) acting as a dehydrogenase in the presence of ubiquinone as proximal electron acceptor and (ii) direct reaction with oxygen as oxidase. A novel assay for the measurement of the oxidase activity was adapted using leuco-dichlorofluorescein-diacetate. Inhibition experiments revealed a striking difference in the susceptibility of DHO-dehydrogenase/oxidase to Brequinar: apparent $K_i = 6.09 \pm 0.05$ (SD) nM (DHO; ubiquinone n = 10), but $K_i = 3.10 \pm 0.09$ (SD) mM (DHO; O_2). Analyses of initial velocity experiments showed non-competitive inhibition of Brequinar with respect to the substrate dihydroorotic acid in both assays (dehydrogenase and oxidase). The inhibitory effect of the latter was compared to that of the competitive inhibitor 5-aza-dihydroorotate (apparent $K_i = 15 \pm 0.25$ (SD) μM). The present kinetic data on the action of the purified rodent DHO-DH with Brequinar and computer-aided analyses provide a better insight into the drug–enzyme interaction.

The dihydroorotate dehydrogenase (DHO-DH†; dihydroorotate-ubiquinone oxidoreductase, EC 1.3.3.1)—the fourth sequential enzyme in the *de novo* biosynthesis of uridine monophosphate—catalyses the conversion of dihydroorotate to the pyrimidine base orotate. DHO-DHases have been isolated from various microorganisms and different mammalian species. These enzymes differ in their requirement for cofactors (FMN, FAD, ubiquinone) and metal ions (Fe^{2+}, Zn^{2+}), and their topochemistry. The enzyme of higher eucaryotes is located on the outer surface of the inner mitochondrial membrane and is intimately linked to the electron transport chain [1]. The enzymes from lower eucaryotes are cytosolic flavoproteins and may react directly with oxygen as electron acceptor (for review see Ref. 2). As pyrimidines are key elements in the biosyntheses of DNA and RNA, a selective inhibition of their biosyntheses has been a strategy in the design and development of antimicrobial, antiparasitic and antitumoral agents, such as the putative anticancer drug Brequinar [3–5]. These investigations and others on cells and mitochondrial particles indicate that DHO-DH is the target for Brequinar, although its structure does not resemble that of known inhibitors. These can be classified into three types: (i) analogs of L-DHO or orotate which may compete for the active site of the enzyme [5–7]; (ii) derivates of naphthoquinones, ubiquinone analogs, that might interfere with electron transport and (iii) inhibitors of the electron transport chain such as cyanide, antimycin or thenoyltrifluoroacetone which do not inhibit the DHO-DH specifically [8]. Efforts have been made to purify mammalian DHO-DH for first characterizations [9] and elucidation of its reaction mechanism [10], but studies on the reaction of purified DHO-DH with drugs such as Brequinar are so far not available but are thought to be of considerable importance since this drug is in clinical trial at present (for review see Ref. 11). Furthermore, since previous reports indicated that DHO-DH has additional oxidase activity [9]—a novel detection method of DHO-dehydrogenase/oxidase on native PAGE has already been described [12]—it was of current interest to investigate and differentiate the influence of Brequinar on both activities.

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† Abbreviations: Brequinar, Brequinar Sodium™, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt, NSC 368390; CoQ_{10}, ubiquinone n = 10; DCIP, 2,6-dichlorophenolindophenol; DHO, dihydroorotate; DHO-DH, dihydroorotate dehydrogenase, dihydroorotate-ubiquinone oxidoreductase; LDCF, leuco-dichlorofluorescein diacetate.

METHODS AND RESULTS

Materials. L-DHO was purchased from the Aldrich Chemical Co. (Gillingham, U.K.), CoQ_{10} was from

Table 1. DHO-DH from rat liver mitochondria

Step	Total protein (mg)	Total activity (U)	Sp. act (U/mg)	Yield (%)	Purification (x-fold)
I	954	0.8	8.39×10^{-4}	100	1
II	763	1.12	1.47×10^{-3}	80	1.8
III	315	0.87	2.76×10^{-3}	33	3.3
IV	0.109	1.04	9.51	0.01	11,300
V	0.054	0.92	17.1	5.7×10^{-3}	20,400
VI	0.016	0.33	20.8	1.7×10^{-3}	24,800

One unit = formation of 1 nmol orotic acid/min.

Kaneka Deutschland (Wiesbaden, F.R.G.) and Brequinar Sodium™ was from Du Pont de Nemours Pharma GmbH (Bad Homburg, F.R.G.). 5-Aza-DHO was obtained from a laboratory synthesis.

Enzyme purification. The DHO-DH was isolated from rat liver in six steps derived in part from a method for bovine DHO-DH [2]. I. Mitochondria were isolated from liver by differential centrifugation. II. Solubilization of mitochondria was via phospholipase treatment (A_2, C) and detergent extraction with Triton X-100 (protein : detergent ratio of 2 : 1). III. Ammonium sulfate precipitation: the solubilized proteins were fractionated by salting out with ammonium sulfate. The precipitate from 20–40% saturation contained DHO-DH. IV. Gel-filtration-chromatography: the dissolved enzyme was applied to a Sephacryl-S300 column and eluted with 50 mM Tris–HCl buffer. V. Ion-exchange chromatography on Q-Sepharose-FastFlow: active fractions were applied in appropriate ionic strength to an ion-exchange column, equilibrated with 20 mM Tris–HCl, 0.1% Triton X-100, pH 9.0. After washing the protein was eluted with a linear salt gradient (0–300 mM KCl). VI. Matrex™ Gel Orange A affinity chromatography: active fractions from the ion-exchange chromatography were desalted on a Sephadex G25 column and applied to a Matrex Gel Orange A column, equilibrated with 50 mM Tris–HCl, 0.05% Triton X-100, pH 9.0. After washing the protein was eluted with a linear salt gradient (0–2.2 M KCl).

Protein determination. Protein concentrations were estimated by the bicinchoninic acid protein assay [13] or according to Lowry. From the purification procedure of DHO-DH (Table 1) a 25,000-fold enrichment of specific activity can be followed. Affinity chromatography resulted in a marked enrichment, but caused a loss in total activity. SDS–PAGE (5% acrylamide stacking and 12% running gel in the discontinuous buffer system of Laemmli) revealed two protein bands for the purified enzyme with apparent molecular masses of 58 (major) and 68 kDa (minor) as seen in Fig. 1. At present it is not clear whether the DHO-DH consists of two subunits, but native PAGE revealed a homogeneous protein and activity band [11].

Enzyme assays and kinetic analysis. The assays are explained in Fig. 2. The reaction mixture of the DCIP dehydrogenase assay—as commonly used for the DHO-DH assay [2, 9, 14]—contained 0.1 mM CoQ_{10}, 0.075 mM L-DHO, 0.02 mM DCIP, 0.1% Triton X-100 in 50 mM Tris–HCl buffer, pH 8.0, and enzyme in the appropriate amount. The loss of absorbance was monitored at 600 nm ($\varepsilon = 18{,}800\ M^{-1}cm^{-1}$). In an alternative assay the produced orotate was measured in the supernatant of acid-precipitated enzyme fractions and monitored at 280 nm ($\varepsilon = 7500\ M^{-1}cm^{-1}$). The oxidase assay [15] contained 10 U/mL horse radish peroxidase, 0.3 mM LDCF—prepared daily—and enzyme in 50 mM Tris–HCl, pH 8.0. For calibration H_2O_2 standards (1–6 nM) were used. The increase in absorbance was monitored at 500 nm ($\varepsilon = 91{,}000\ M^{-1}\ cm^{-1}$). The dehydrogenase and oxidase assays were linear with respect to the amount of protein and time up to at least 15 min. Apparent Michaelis constants (K_m) for the substrate L-DHO were estimated from non-linear fits to a rectangular hyperbola, the Michaelis–Menten equation, of the initial rates of velocity at various substrate concentrations (0.3–750 μM). The values were $K_m = 6.08 \pm 0.75$ (SD) μM, $V_{max} = 0.71 \pm 0.02$ μM (DHO; CoQ_{10}) and $K_m = 0.04 \pm 0.01$ (SD) μM, $V_{max} = 0.47 \pm 0.02$ μM (DHO; O_2). The DHO-DH from rat liver has an additional oxidase activity of about 15% in the absence of CoQ_{10}.

Inhibition of DHO-DH. At two different substrate concentrations (10 and 75 μM L-DHO) the inhibitor concentration was varied from 0–75 μM in the case of the dehydrogenase assay (CoQ_{10}, DCIP) and from 0–5 mM Brequinar in the case of the oxidase assay (O_2, LDCF). In control experiments an interaction of the Brequinar molecule with DCIP or LDCF, which could falsify the measurement of the change of absorbance, was not found. Estimation of apparent inhibitor constants from Dixon plots (the plot of the reciprocal initial velocity versus Brequinar concentration at different substrate concentrations) gave the following results: (i) $K_i = 6.09 \pm 0.05$ nM for the dehydrogenase reaction and (ii) $K_i = 3.10 \pm 0.09$ mM for the oxidase reaction. Brequinar proved to be a non-competitive inhibitor with respect to the substrate L-DHO. Inhibitory studies on the substrate analog 5-aza-DHO (0–1 mM) revealed an apparent $K_i = 15 \pm 0.25$ μM and a competitive type of inhibition with respect to the substrate L-DHO, as was found with the enzyme of other species [7, 16]. The extremely low inhibitor constant ($K_i = 6.09$ nM), which is in line with data on solubilized mitochondria [3, 4], supports the specific action of

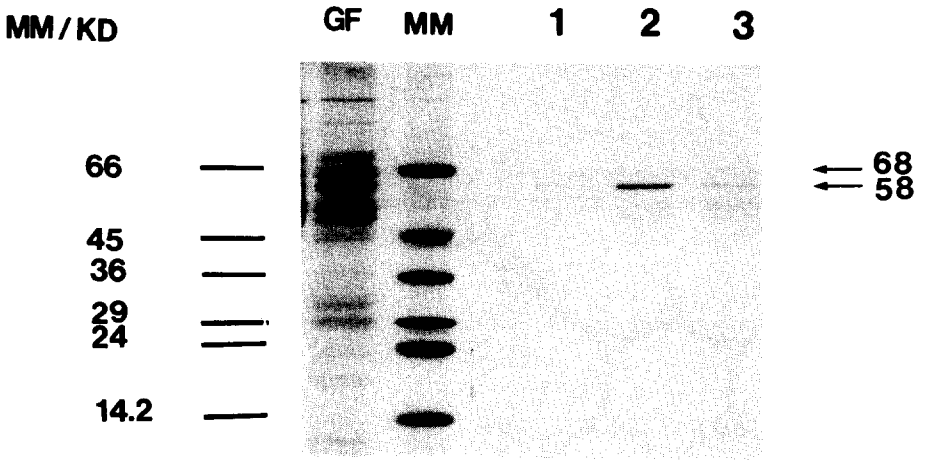

Fig. 1. SDS–PAGE of the purified rat liver DHO-DH. Apparent molecular weights: 58 kDa (major), 68 kDa (minor). Protein was stained with Coomassie blue; as molecular weight marker (MM) Dalton Mark VII-L™ kit (the Sigma Chemical Co., Deisenhofen, F.R.G.) was used, GF, gel-filtration fraction; 1–3, successive chromatographic fractions of purified DHO-DH (step V).

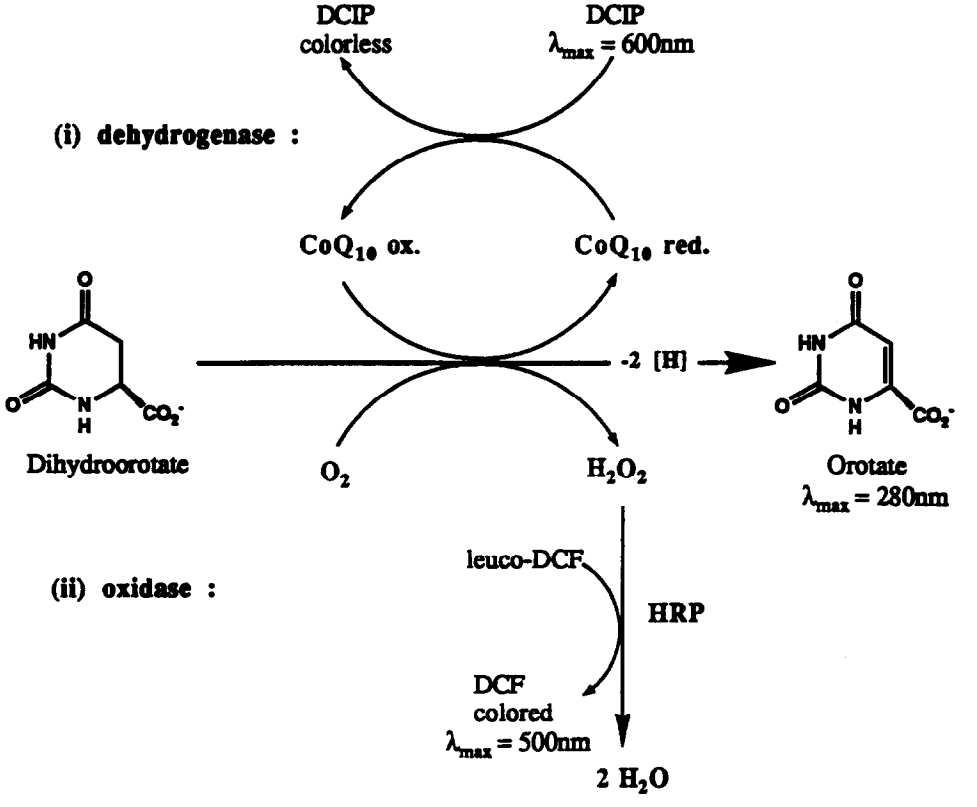

Fig. 2. The formation of the pyrimidine base orotate from dihydroorotate due to the two possible modes of catalytic action of the DHO-dehydrogenase/oxidase is described: (i) dehydrogenase reaction and DCIP assay; (ii) oxidase reaction and LDCF assay. HRP, horse radish peroxidase.

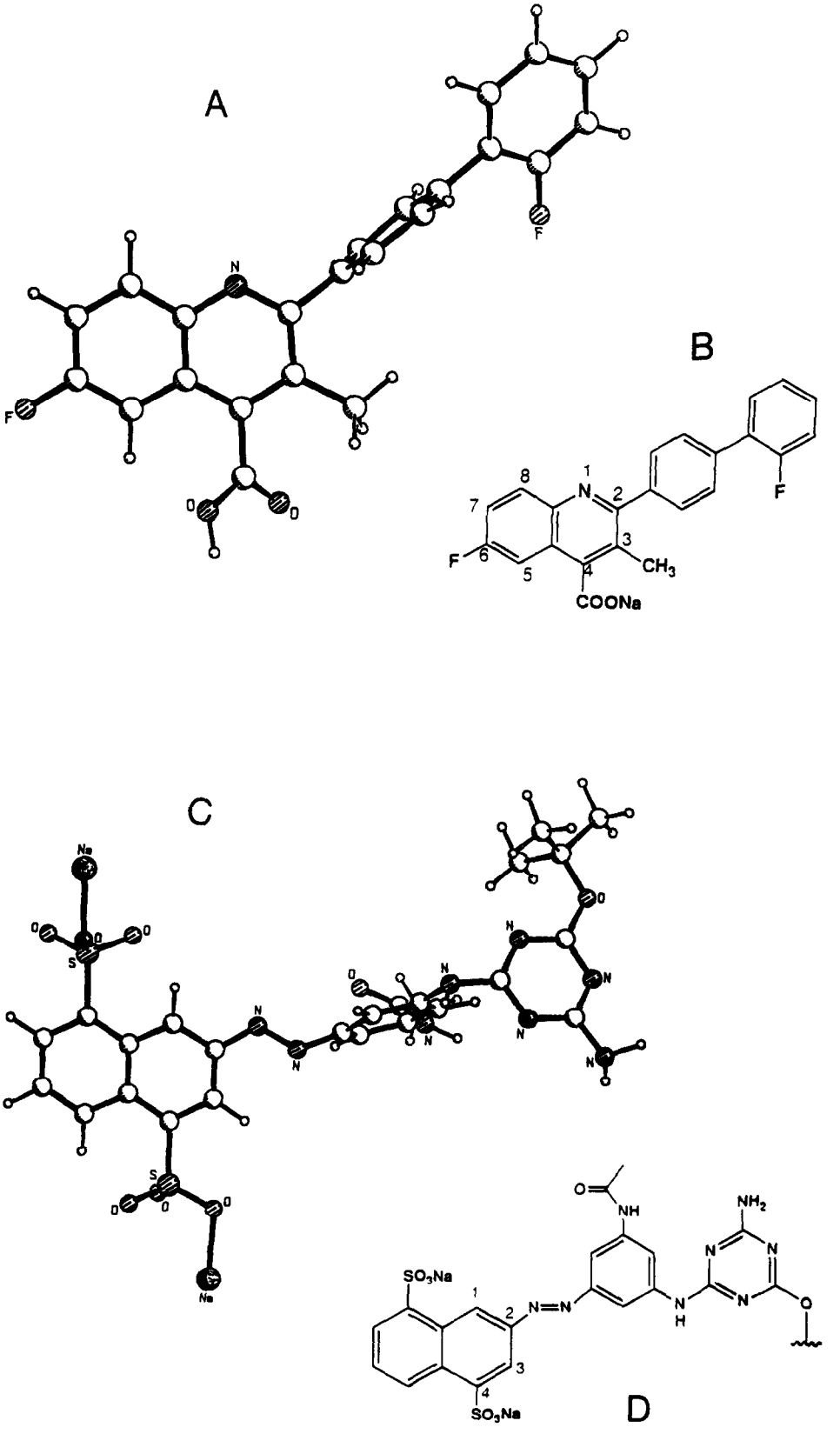

Fig. 3. Comparison of the structures of Brequinar and the dye ligand Matrex Gel Orange A. (A) 3D structure of Brequinar (Schakal plot) as obtained by the MM2 force field implemented in MakroModel. (B) Structural formula of Brequinar. (C) 3D structure of the dye ligand. (D) Structural formula of the dye ligand.

Brequinar. From the 10^6-fold weaker inhibitory action a specific influence of Brequinar on the oxidase activity of the DHO-DH can hardly be deduced.

Analyses of the three-dimensional structure. During the development of the purification procedure different dye affinity ligands were tested, but only the dye Orange A bound the rodent DHO-DH. This ligand revealed similarities in the structure of Brequinar and thus was investigated with the aid of theoretical studies: Fig. 3 shows Schakal plots and the chemical structure of Brequinar (A, B), and the dye affinity ligand Matrex Gel Orange A (C, D). The geometries were optimized using the MM2 force field implemented in MakroModel (MakroModel V3,1X). Brequinar (Fig. 3A, B) consists of a naphthalene-like quinoline ring with an anionic center at the C(4) position. The quinoline ring is substitued with a biphenyl system at C(2) and the first ring is oriented perpendicularly to the naphthalene plane (torsion angle 70°). The second ring is nearly in the naphthalene plane. The dye ligand Matrex Gel Orange A (Fig. 3C, D) shows the same substitution pattern at its naphthalene system: there is an anionic center, the sulphonyl group, at C(4); at C(2) it is linked to a phenyl ring by a diazo-group and the ring plane is likewise almost perpendicularly orientated to the naphthalene system. The second ring (triazole) lies in the plane of the molecule. Thus, the molecular mechanistic analysis showed good agreement in the distribution and arrangement of the hydrophobic and charged regions of the two molecules.

DISCUSSION

The nature of the drug–enzyme interaction may be deduced from the molecular mechanistic studies of the affinity ligand and the inhibitor molecule. The present theoretical analysis verifies that the very effective binding of the rat liver DHO-DH to Matrex Gel Orange A during the chromatography procedure is based on a specific structural and electrostatic arrangement of the molecule which resembles the molecular and electronic structure of Brequinar very closely. This conclusion is supported by studies on the structure–activity relationship of quinoline carboxylic acids [17]. This study identified three critical regions for the binding of Brequinar and analogs: the C(2) position with bulky hydrophobic substituents; a carboxylic acid at the C(4) position and the benzo-portion of the quinoline ring. Due to these stereoconformities a binding of high specificity of the enzyme protein to Matrex Gel Orange A was to be expected and likewise demonstrated, since an elution of the enzyme from the affinity ligand was achieved under enforced conditions (1.5 M KCl only) with a loss in total activity. Thus, it is not too unreasonable to assume that the very potent action of Brequinar on the dehydrogenase activity is due to its stereoconformation and charge distribution.

At present the 3D analyses give no insight into the binding site of Brequinar. This receives more elucidation from our kinetic data. The differential Michaelis constants $K_m = 6.08\,\mu M$ (CoQ_{10}) and $K_m = 0.04\,\mu M$ (O_2) give evidence that the dehydrogenase and oxidase reactions follow different reaction mechanisms. Concerning the dehydrogenase reaction, it is known from bovine liver DHO-DH [10] that the reaction follows a non-classical ping-pong mechanism, in the presence of an ubiquinone cosubstrate, with two physically separated catalytic sites: one site for the binding of the substrate L-DHO and the other for ubiquinone. With respect to this model, it may be surmised that the drug interacts with the ubiquinone binding site of the DHO-DH, which is of consequence for the dehydrogenase but has marginal influence on the oxidase reaction. The oxidase reaction mechanism has not yet been established but may be of radical type.

The physiological consequence of the oxidase activity of DHO-DH, with respect to drug treatment and in view of the possible conversion of dehydrogenase to oxidase under certain pathological conditions [11, 12, 18], remains to be investigated.

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