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Structural and Functional Comparison of Agents Interfering with Dihydroorotate, Succinate and NADH Oxidation of Rat Liver Mitochondria

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ABSTRACT. Mitochondrially bound dihydroorotate dehydrogenase (EC 1.3.99.11) catalyses the fourth sequential step in the *de novo* synthesis of uridine monophosphate; this enzyme uses ubiquinone as the proximal and cytochrome oxidase as is the ultimate electron transfer system. Here, seven compounds with proven antiproliferative activity and *in vitro* antipyrimidine effects were investigated with isolated functional mitochondria of rat tissues in order to differentiate their anti-dihydroorotate dehydrogenase potency versus putative effects on the respiratory chain enzymes. Ten μM of brequinar sodium, the leflunomide derivatives A77–1726, [2-cyano-3-cyclopropyl-3-hydroxy-enoic acid (4-trifluoromethylphenyl)-amide], MNA 279, (2-cyano-*N*-(4-cyanophenyl)-3-cyclopropyl-3-oxo-propanamide), MNA715 (2-cyano-3-hydroxy-*N*-(4-(trifluoromethyl)-phenyl-6-heptanamide), HR325 (2-cyano-3-cyclopropyl-3-hydroxy-*N*-[3′-methyl-4′-(trifluoromethyl)phenyl]-propenamide), and the diazine toltrazuril completely inhibited the dihydroorotate-induced oxygen consumption of liver mitochondria. Succinate and NADH oxidation were found to be influenced only at elevated drug concentration (100 μM), with the exception of HR325, 10 μM of which caused a 70% inhibition of NADH and 50% inhibition of succinate oxidation. This was comparable to the effects of toltrazuril, which caused an approximate 75% inhibition of NADH oxidation. Ciprofloxacin was shown here to have only marginal effects on the redox activities of the inner mitochondrial membrane. This differentiation of drug effects on mitochondrial functions will contribute to a better understanding of the *in vivo* pharmacological activity of these drugs, which are presently in clinical trials because of their immunosuppressive, cytostatic or anti-parasitic activity. A comparison of the influence of A77-1726, HR325, brequinar and 2,4-dinitrophenol on energetically coupled rat liver mitochondria revealed only a weak uncoupling potential of A77-1726 and brequinar. In addition, a modeling study was raised to search for common spatial arrangements of functional groups essential for binding of inhibitors to dihydroorotate dehydrogenase. From the structural comparison of different metabolites and inhibitors of pyrimidine metabolism, a 6-point model was obtained by conformational analysis for the drugs tested on mitochondrial functions, pharmacophoric perception and mapping. We propose our model in combination with kinetic data for a rational design of highly specific inhibitors of dihydroorotate dehydrogenase. BIOCHEM PHARMACOL **56**;8:1053–1060, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. dihydroorotate dehydrogenase; respiratory chain; isoxazol; cinchoninic; diazine-derivatives; modeling study

The functional link of pyrimidine *de novo* synthesis to the respiratory chain in the inner mitochondrial membrane at the stage of DHOdehase‡ (EC 1.3.99.11) seems to be the result of an extraordinary compartmentation that is evolutionarily preserved in higher eukaryotic cells (Scheme). DHOdehase catalyzes the conversion of dihydroorotate to orotate with ubiquinone as the proximal and cytochrome oxidase with molecular oxygen as the final electron-acceptor system. The other five enzymes of the pathway are present in the cytosol of cells [1–3]. Because pyrimidine nucleotides serve essential functions in nucleic acid metabolism and sugar nucleotide formation for glycosylation of proteins and lipids, a decrease in one of the six enzyme activities might make it the rate-limiting reaction in the de novo pathway and might limit the pools of pyrimidine nucleotides in rapidly dividing cells.

Interest in inhibitors of DHOdehase has greatly increased as chemotherapeutic agents to reduce aberrant immunologic reactions [4, 5] and to interfere in the

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‡ *Abbreviations:* A77-1726, 2-cyano-3-cyclopropyl-3-hydroxy-enoic acid (4-trifluoromethylphenyl)-amide; ACI, acceptor-control index; DHOdehase, dihydroorotate dehydrogenase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HR325, 2-cyano-3-cyclopropyl-3-hydroxy-*N*-[3′-methyl-4′-(trifluoromethyl)phenyl]-propenamide; MNA 279, 2-cyano-*N*-(4-cyanophenyl)-3-cyclopropyl-3-oxo-propanamide; MNA715, 2-cyano-3-hydroxy-*N*-(4-(trifluoromethyl)-phenyl-6-heptanamide; P/O ratio, phosphate bound per O_2 consumed; and RCR, respiratory control ratio.

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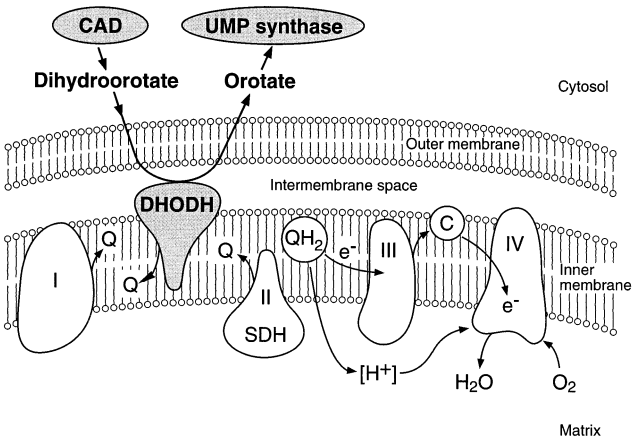

SCHEME

multiplication of animal parasites and parasitic protozoa in malaria-infected individuals and pathogens of opportunistic diseases [6]. Inhibitory compounds of DHOdehase can be classified into three groups: Group 1, analogs of L-dihydroorotate, orotate or quinone analogs [7–10]; Group 2, compounds with an unknown mode of interference with DHOdehase catalysis, such as derivatives and analogs of cinchoninic acid (brequinar sodium [11]), isoxazol (leflunomide [12]), diazine (toltrazuril [13]) or others (ciprofloxacin [14]); Group 3, inhibitors of the electron transport chain such as cyanide or azide, which would not interfere with the isolated enzyme *in vitro* but would impede the flow of electrons to the final acceptor molecular oxygen [1, 15, 16]. The affinity dye ligand Matrex gel Orange A used in chromatography of dehydrogenases, especially in purification protocols for the flavoprotein DHOdehase from animal tissues [17, 18], apparently closely mimics the stereochemical conformation of the Group 2 drug brequinar [19].

The pharmacological and clinical profiles of numerous Group 2 compounds have been described in the literature. Brequinar, which originally was discovered as a cytostatic agent with a strong effect on DHOdehase and subsequent reduction of pyrimidine nucleotide pools, is currently being evaluated for its effectiveness as an immunosuppressive agent for preventing graft rejection [5]. Leflunomide, with the anti-DHOdehase active metabolite A77-1726, is currently in clinical phase III trials for the treatment of rheumatoid arthritis after phase II studies assessed its safety and effectiveness [20]. This disease is believed to have an underlying autoimmune pathology. The immunomodulating action of leflunomide has been correlated with the prominent effect of A77-1226 on pyrimidine de novo synthesis, which is necessary for clonal expansion of immuno cells, especially of T lymphocytes [21, 22]. Other isoxazol derivatives, MNA715 and MNA279, have been found to block rejection after allograph and xenograph transplantation in animal models [23]. Toltrazuril (Baycox^{TM}) is used as a broad-spectrum anticoccidal (*Eimeria falciformis* and *E. tenella*) drug. NADH:ubiquinone oxidoreductase, also known as complex I of the respiratory chain, was pinpointed as the principal but not the sole intramitochondrial target for toltrazuril [13]; effects on DHOdehase activity were shown in histochemical studies [16]. Ciprofloxacin, which was found to decrease purine and pyrimidine nucleotide pools in cells, was surmised to interfere with components of the mitochondrial respiratory chain, similar to dichloroallylawsone [10], rather than to interfere directly with pyrimidine *de novo* synthesis [14].

In view of the diverse structures and applications of the second group of compounds and poor knowledge of their mode of action, the present study was carried out in order to search for common spatial arrangements of functional groups essential for binding to DHOdehase. Thus, we coupled our inhibition studies with computer-assisted analysis of the structural features of effective and potentially effective compounds. Because rodents and especially rats are preferred animal models for *in vivo* testing and investigation of pharmacological drugs, we chose rat tissue for experiments to collate the second group of compounds for their effect on the mitochondrially bound DHOdehase activity. Because of the known activity of the drug toltrazuril to block the respiratory chain, it was the rationale of the present study to apply the same kind of assay and test system for the evaluation of putative effects of the other drugs on prominent redox functions of the mitochondrial inner membrane. This has not been investigated before.

MATERIALS AND METHODS

Materials

L-Dihydroorotate, sodium cyanide, rotenone, malonate, and succinate were purchased from Sigma; NADH was from Boehringer Mannheim. Tissues from male Wistar rats (age 3–5 months) were kindly provided by Prof. Dr. W. Wesemann, Neurochemistry, School of Medicine, Marburg, Germany.

Methods

Unless otherwise stated, all chemicals were from Boehringer, Serva, Merck or Sigma, at the purest grade available. A77-1726, MNA 279, MNA715, and HR325 were obtained from Hoechst–Marion Roussel Deutschland GmbH. Brequinar sodium (NSC 368390, DuP 785) was obtained from DuPont Pharma GmbH; toltrazuril (Baycox^{TM}) and ciprofloxacin were from Bayer AG Leverkusen (see Fig. 1).

Preparation of Mitochondria

Tissues prepared from bled animals (3–5 were combined) were transferred to the appropriate isolation medium at 4^{o} and processed according to standard methods [24, 25]. For liver the medium contained: 250 mM mannitol, 10 mM Hepes/KOH, 1 mM EGTA, pH 7.4; for kidney it contained: 250 mM sucrose, 3 mM Tris/HCl, 1 mM EDTA, pH 7.4. The protocol for isolation of heart mitochondria included 5 U/mg of subtilisin for tissue destruction. Following resus-

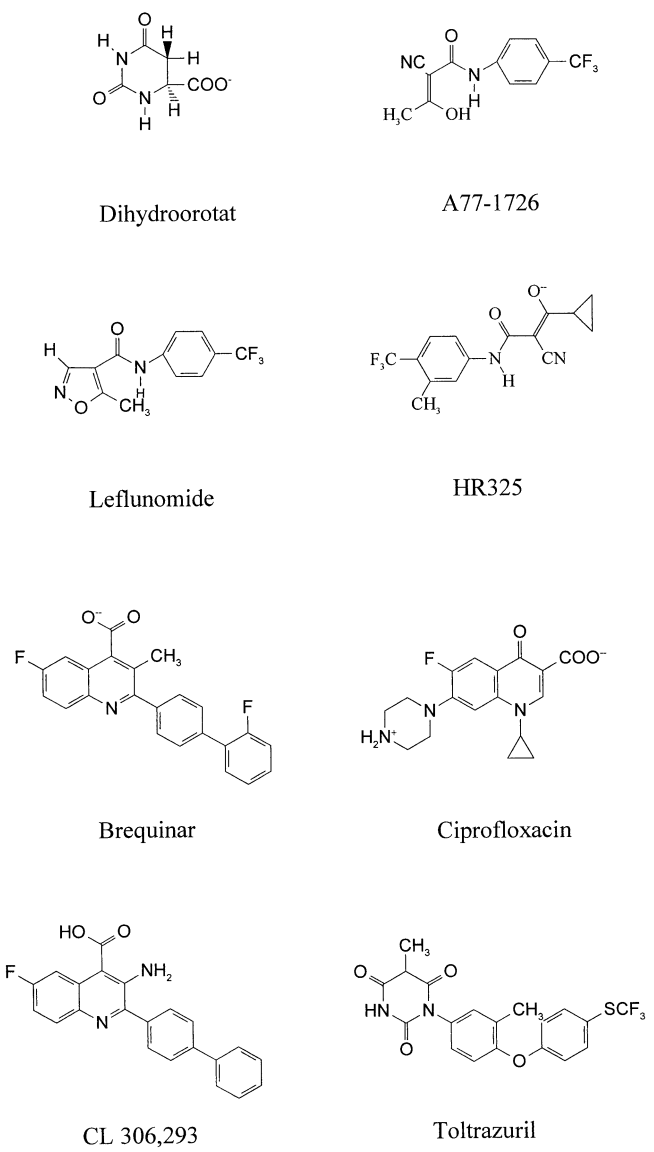

FIG. 1. Two-dimensional depictions of structures. Dihydroorotate, A77–1726, HR325, leflunomide, brequinar, ciprofloxacin, CL 306.293 and toltrazuril.

pension of mitchondria in a mixture of 210 mM mannitol, 70 mM sucrose, 0.1% BSA, 10 mM Tris/HCl buffer pH 7.4, they were purified on 5 mL of Percoll (Pharmacia Upjohn) containing 200 mg of mannitol and 125 mg of sucrose. The mitochondrial fraction was kept on ice and used for the assays of oxygen consumption and electron transport the day of preparation.

Enzyme Activities

The organelles in the mitochondrial fractions obtained and handled by the routine protocols are "broken" mitochondria, due to the lack of osmotic support for intactness of the membranes and as evidenced by high NADH oxidation (see Results). These were taken here to evaluate the effect of drugs on the oxidation of dihydroorotate, NADH and succinate, without limitation on transport or uptake.

The protein content of mitochondrial fractions was determined using the Lowry-Folin method with BSA as standard protein. For a reliable determination of dihydroorotate oxidation, the protein content was adjusted to 5 mg/50 μL. The incubation vessel contained 50 μL of mitochondria in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4 and was connected to a Clark-type electrode (Oxylab, Biolytik) and equilibrated for 2 min at 30°. After addition of 5 mM L-dihydroorotate, oxygen consumption induced by mitochondrial cytochrome oxidase was recorded and calculated by the Oxylab 1.81b program. Without substrate the oxygen consumption was negligible. DHOdehase activity was calculated with respect to 1 mol of dihydroorotate per 1/2 mol of dioxygen (1 mol O). The determination of NADH and succinate oxidation was performed similarly, but adjustment of the samples was 1.0–1.5 mg of protein/50 μL. Measurement of cytochrome-oxidase (complex IV) was performed in the presence of 7 mM ascorbic acid, 0.7 mM tetramethyl-p-phenylendiamin-dihydrochloride, and 0.3 μM cytochrome c in the same buffer as described previously [26]. To test the influence of agents on dihydroorotate-induced oxygen consumption, 10 μL of buffered solution was added to give final concentrations of 0.1, 1 μM, and 10 μM NADH or succinate oxidation was determined in the presence of 10 and 100 μM of the agents with liver mitochondria and in the presence of 50 and 100 μM with heart and kidney mitochondria. For comparison, the complete inactivation of the flux of electrons was achieved upon addition of 1 mM sodium cyanide as inhibitor of the terminal step, cytochrome oxidase.

Preparation and Assay of Functionally Coupled Mitochondria

In order to study functional coupling of electron transport to oxidative phosphorylation, liver mitochondria were isolated using the medium described above, but without BSA. After centrifugation, the upper layer of the mitochondrial fraction containing small and mitochondrial particles was discarded. Mitochondria were used only within 1 hr to assay oxygen consumption. The incubation medium contained 5 mM potassium phosphate buffer (pH 7.5, 25°), 5 mM $MgCl_2$, 2.5 μM rotenone (to block endogenous complex I activities) and 50 μL of mitochondria. The reaction was started by addition of 5 mM succinate (state 4 respiration). After 2 min, the addition of 0.1 mM ADP caused an increase in oxygen consumption (state 3). Excess addition of ADP caused a further increase in oxygen consumption. The respiration rate in the presence of excess ADP versus the respiration rate without ADP is defined as the acceptor control index (ACI). This ratio describes the intactness of mitochondria; a value of 5 through 10 indicates mitochondria in good or very good condition. To characterize the functional coupling of mitochondria, the evaluation of the ACI, the P/O ratio (ATP synthesized from ADP and phosphate divided by oxygen consumed in state 3 respira-

tion) and the RCR (respiration rate in the presence of succinate plus a limited amount of ADP divided by respiration rate without ADP) were evaluated as described by Rickwood *et al.* [24]. If the addition of ADP in the presence of an agent did not cause mitochondria to elevate the oxygen consumption, an uncoupling effect could be suspected on the assumption that ATP formation and transport were not affected. The elevation of oxygen consumption with succinate (control state 4) on addition of an agent would underline the uncoupling features of such a compound.

Molecular Modeling

All work was performed on Silicon Graphics workstations with the Sybyl program version 6.1 [27]. The experiments started with a data set of 20 structures known as cell metabolites or metabolic inhibitors (see below). The molecules were sketched within Sybyl. Three-dimensional structures were generated by the CONCORD program [28] and further used as starting geometries in a conformational analysis using the MULTISEARCH modul. Pharmacophore perception and mapping was done by the DISCO modul [29].

RESULTS

Structural Comparison

Initially, a set of 20 molecules that are generally relevant to pyrimidine metabolism and enzymes were subjected to this study: 1. dihydroorotate, 2. orotate, 3. aza-dihydroorotate, 4. aza-orotate, 5. spiro-cyclopropanbarbiturate [30], 6. benzylhydantoin [30], 7. carbamoyl-aspartate, 8. pteridine, 9. methothrexate, 10. riboflavin, 11. ubiquinone (N = 1), 12. dichloroallyllawsone, 13. ciprofloxacin, 14. toltrazuril, 15. leflunomide, 16. HR325, 17. A 77-1726, 18. brequinar, 19. CL306.293, 20. Matrex-Gel Orange A. After preliminary trials with the DISCO program, it became clear that the numbers and the spatial distribution of the functional groups of the substrate and product analogs and of the other compounds chosen for comparison differed from the corresponding pattern found for the DHOdehase inhibitors of unknown type. Therefore, we focused our attention upon seven drugs classified above as Group 2 compounds. These compounds are presented as two-dimensional depictions in Fig. 1.

Functional groups common to all structures are two hydrogen bond acceptor atoms, one hydrogen bond donor atom, and three hydrophobic centers. The definition for hydrophobic centers was expanded to include methyl-, trifluoro-methyl- as well as cyclopropyl groups. In addition, one site point for hydrogen bond acceptors and three site points for hydrogen bond donors were considered as common pharmacophoric points. Leflunomide was chosen as template structure because it has the fewest pharmacophoric points and the least flexible structure. DISCO found a pharmacophoric mapping of 6 points at a tolerance of 2.2 angstroms between pharmacophoric points. This model included two hydrogen bond acceptor atoms, three hydrophobic centers and one hydrogen bond donor site point. The superposition of all structures using these points gives an average RMS fit of 1.47 angstroms. In this superposition, all structures are roughly oriented in a plane. Ciprofloxacin only partially fits to this model due to the missing biphenyl group ("hydrophobic tail") and the alkylated quinoline nitrogen, acting as hydrophobic centers and acceptor atom in the cases of brequinar and CL306.293, respectively. A schematic representation of this model is presented in Fig. 2. A three-dimensional representation of this model is shown in Fig. 3, where conformers of A77-1726 and brequinar were superimposed using the six pharmacophoric points. Figure 4 shows the corresponding superposition with conformers of A77-1726 and Toltrazuril. The 6-point model could be expanded to a 10-point model including all common pharmcophoric points, if we allowed for a wider tolerance. The 10-point model showed a maximum tolerance of 4 Å between pharmacophoric points and an average RMS fit of 1.87 Å.

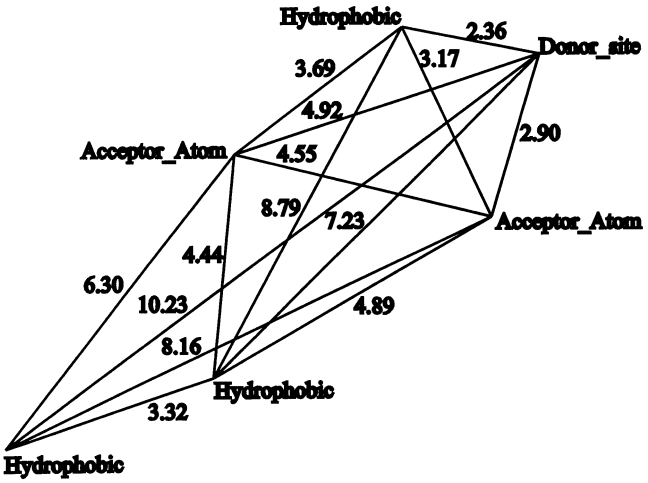

FIG. 2. Schematic representation of the 6-point model. Each corner represents the location of a pharmacophoric point and is labeled with the class to which the point belongs. The lines connecting the points are labeled with the distance between the points in angstroms. The tolerance was ±2.20 Å.

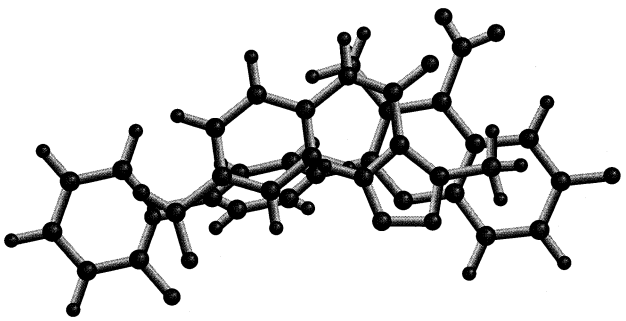

FIG. 3. Superposition of brequinar and A77-1726 generated using the 6-point model.

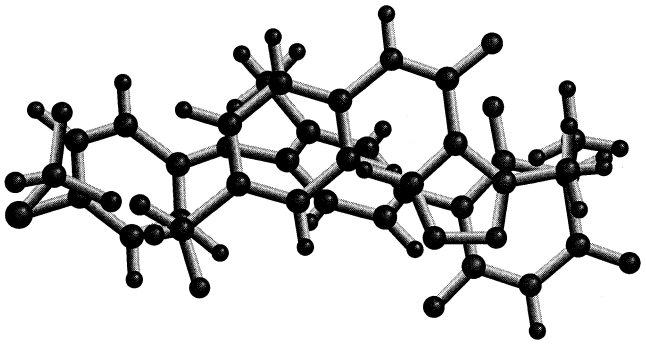

FIG. 4. Superposition of toltrazuril and A77-1726 generated using the 6-point model.

Assay of Dihydroorotate, NADH and Succinate Oxidation

To measure dihydroorotate-, NADH- or succinate-dependent oxygen consumption, we used standard techniques. When referred to the total protein content of liver mitochondria, the DHOdehase activity in preparations from 12 animals varied from 1.4–3.1 nmol O/min · mg. This was in the same range as previously reported, i.e. 5.4 nmol O/min · mg, for quite an other set of experiments using other oxygen-monitoring equipment. The respiratory activities of liver mitochondria preparation with NADH (35 nmol O/min · mg) and succinate (17 nmol O/min · mg) were higher than with dihydroorotate and were similar to those reported by others [25, 31]. The oxidation of reduced cytochrome c was 1,400 nmol O/min · mg [26]. This led us to conclude that cytochrome oxidase was not rate-limiting for the measurements. In addition, we found the activity to be unaltered by any of the agents under study (see below) except sodium cyanide, which also abolished the oxygen consumption induced by dihydroorotate, NADH and succinate.

Drug Effects on Dihydroorotate, NADH and Succinate Oxidation

Table 1 summarizes the results obtained after administration of 0.1, 1.0, and 10 μM A77-1726, MNA715, MNA279, HR325, brequinar, toltrazuril and ciprofloxacin on mitochondria, in relation to the control reaction (without agents) of the same preparation. A prominent decrease in rat liver DHOdehase activity was observed with brequinar, 0.1 through 1 μM (90% inhibition). Only 20% inhibition was observed on addition of 1 μM ciprofloxacin (80% residual activity in Table 1). Because 10 μM was found to be only marginally more effective, and because the drug did not really fit into the pharmacophoric model, higher concentrations were not tested in the present study. A moderate reduction in enzyme activity was observed on addition of 1 μM toltrazuril followed by a steep decrease at 1 through 10 μM. From the 27%, 26% and 24% residual DHOdehase activity (*ca.* 75% inhibition) observed with 1 μM isoxazol derivatives MNA715, MNA279 and HR325, a similar effect of these compounds on DHOdehase was deduced. This was close to the 35% residual activity of mitochondrially bound DHOdehase in the presence of 1 μM A77–1726.

Table 2 shows the influence of the compounds tested at concentrations of 10 and 100 μM on the oxidation of NADH and succinate. In comparison to the others, toltrazuril and HR325 were the strongest inhibitors of NADH oxidation. Residual NADH oxidation in the presence of 10 μM toltrazuril was 26% (73% inhibition), while in the presence of HR325 it was 30% of control (70% inhibition). The same concentration of A77-1726 caused marginal if any reduction in NADH and succinate oxidation; a 33% reduction was observed with 100 μM of this drug. Similar effects of brequinar and MNA279 were seen on NADH and

TABLE 1. Effect of compounds upon oxidation of dihydroorotate by rat liver mitochondria

Compound Concentration	Dihydroorotate oxidation [% of control]*		
	0.1 μM	1 μM	10 μM
A 77-1726	55	35	0
MNA715	32	27	0
MNA279	42	26	0
HR325	30	24	0
Brequinar	70	10	0
Toltrazuril	100	72	0
Ciprofloxacin	100	80	75

*Enzyme activity: mean activity of dihydroorotate oxidation in liver mitochondria was 2.5 nmol O/min · mg (N = 7). SD was maximally 10% of the mean. [1 nmol O/min · mg] equivalent to [1×10^{-3} U/mg].

Enzyme activity (*) was assayed by following oxygen consumption by mitochondria (5 mg of protein) in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4 at 30°. Following the equilibration for 2 min in the vessel, 5 mM dihydroorotate was added. For evaluation of drug effects, the samples were incubated with the compounds for 1 min before addition of the substrate. Percentage of inhibition was related to the control (100% activity). Determination in triplicate.

TABLE 2. Effect of compound upon the oxidation of NADH and succinate by rat liver mitochondria

Compound Concentration	NADH oxidation [% of control]		succinate oxidation [% of control]	
	10 μM	100 μM	10 μM	100 μM
A 77-1726	96	77	95	67
MNA715	100	64	100	77
MNA279	100	40	100	67
HR325	30	12	48	16
Brequinar	100	50	100	61
Toltrazuril	26	7	100	78
Ciprofloxacin	100	91	100	95

*NADH oxidation in rat liver mitchondria was 35 nmol O/min · mg (N = 7); succinate oxidation was 17 nmol O/min · mg (N = 7). SD was maximally 10% of the mean.

Activities were assayed by following the oxygen consumption of mitochondria (1 − 1.3 mg of protein) in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4 at 30°. Following equilibration for 2 min in the vessel, 20 mM NADH or 20 mM succinate, respectively, were added (*). For evaluation of drug effects, the samples were incubated with the compounds for 1 min before addition of the substrate. Percentage of inhibition was related to control (100% activity). Mean of two separate experiments, determination in triplicate.

TABLE 3. Effect of compounds upon oxidation of NADH and succinate in rat heart and kidney mitochondria

Compound Concentration	NADH oxidation [% of control]		succinate oxidation [% of control]	
	50 μM	100 μM	50 μM	100 μM
	heart*		heart†	
A 77-1726	85	42	91	67
HR325	5	0	15	17
Brequinar	70	72	73	61
	kidney*		kidney†	
A77-1726	56	37	88	74
HR325	8	6	28	28
Brequinar	90	50	100	86

*NADH oxidation in heart mitochondria was 630 nmol O/min · mg; in kidney mitochondria 190 nmol O/min · mg.

†Succinate oxidation in heart mitochondria was 90 nmol O/min · mg; in kidney mitochondria 70 nmol O/min · mg. SD was maximally 10% of the mean.

Activities were assayed by following oxygen consumption by mitochondria (0.1 − 0.15 mg of protein) in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4 at 30°. Following equilibration for 2 min in the vessel, 20 mM NADH or 20 mM succinate, respectively, were added. For evaluation of drug effects, the samples were incubated with the compounds for 1 min before addition of the substrate. Percentage of inhibition was related to the control (100% activity). Mean of two separate experiments, determination in triplicate.

succinate oxidation at 100 μM. Ciprofloxacin (last column) did not significantly affect complex I or complex II. As can be seen, the most potent inhibitor of succinate oxidation was HR325 (*ca.* 50% reduction by 10 μM and 30% reduction by 1 μM, not reported in Table 2). One hundred μM A777-1726 or MNA715 or MNA279 or brequinar reduced the oxidation of added succinate to a similar extent. The low effect of 100 μM toltrazuril on succinate oxidation agrees with the observations of Harder and Haberkorn [13].

To verify the prominent effect of HR325 on the respiratory chain activities and to exclude a tissue-specific effect, kidney and heart mitochondria were isolated and tested in the same manner. The effects of HR325, A77-1726 and brequinar on succinate oxidation by heart and kidney mitochondria were similar to those observed with liver mitochondria (Table 3). The effect of isoxazol derivatives on NADH oxidation by heart and kidney mitochondria was found to be slightly greater than liver mitochondria (Table 2). The data of Table 3 confirm that HR325 significantly interfered with NADH and succinate oxidation, even at low concentration.

Influence of A77-1726, HR325 and Brequinar on Coupled Mitochondria

Physicochemically, the isoxazol derivatives and brequinar are moderately weak acids coupled to a relatively bulky, lipophilic, electron-withdrawing residue, a structure that is common among chemicals with potential uncoupling activity. Such agents have not been shown to chemically alter functional proteins, but have been known to increase conductivity across phospholipid bilayer membranes by acting as proton ionophores. Thus, we wanted to discriminate the effects of these compounds as inhibitors of the respiratory chain from their possible effects as uncouplers of the respiratory phosphorylation. Because uncoupling of mitochondria involves changes in a number of interrelated parameters, criteria describing the intactness of isolated mitochondria had to be strictly controlled [24, 25].

The following data are expressed as means and standard error for 7 separate mitochondria preparations with each determination in triplicate: ACI, 7.69 ± 1.20; P/O ratio 2.11 ± 0.19; respiratory control ratio, 5.16 ± 0.55. Whereas the P/O ratio was found to be unaffected by 1 or 10 μM A77-1726 and 1 or 10 μM brequinar, respectively, 10 μM HR325 released the respiratory control: the ADP-induced elevation of oxygen consumption (state 3, for the formation of ATP from ADP and phosphate) did not return to state 4 (basic respiration without ADP). Because the same concentration of HR325 caused considerable inhibition of the electron transport chain (Table 2, by 50–70%), we eliminated it from further studies.

We next compared the oxygen consumption of intact mitochondria in the absence (control = 100%) and presence of A77-1726 or brequinar or 2,4-dinitrophenol. Under our conditions, the increase in oxygen consumption was 660% induced by 50 μM 2,4-dinitrophenol, 26% by 50 μM A77-1726 and 66% by 50 μM brequinar (mean of two separate preparations, each determination in triplicate). These values could point to a putative "uncoupling potential" of the two drugs. However, because of a more pronounced inhibitory effect of brequinar on succinate respiration (Table 2), the "uncoupling potential" of brequinar, determined under the present conditions to be twice that of A77-1726, could have been underestimated.

The compound 2,4-dinitrophenol, a widely used "classic" uncoupler applied in the range of 1×10^{-5} M, was usually considered to be of low potency, i.e. comparable to that of dicumarol (1×10^{-5} M) and oleic acid (5×10^{-5} M), a "nonclassical" uncoupling compound [32]. In contrast to this group of compounds, derivatives of chlorophenylhydrazone (FCCP) or salicylanilide (S-13) were found to operate in the range of 10^{-7}–5×10^{-9} [32]. With respect to this ranking, the isoxazol derivative A77-1726 and the cinchoninic acid derivative brequinar could be categorized as compounds with only very weak uncoupling potential.

DISCUSSION

The present study examined potent DHOdehase inhibitors that are superficially unrelated to the substrates and have been discovered and developed in different laboratories. This approach is mandated by the current lack of a three-dimensional structure of the mammalian DHOdehase, which would be obtained from x-ray analysis. The crystal structure of the DHOdehase A from *Lactococcus lactis* has been recently determined [33]; however, this protein belongs to another protein family of dihydroorotate-oxidizing enzymes that are not coupled with the electron transport system.

Our pharmacophoric model defines a new class of compounds within the Group 2 DHOdehase inhibitors: isoxazol derivatives, cinchoninic acid derivatives, diazine derivatives [17–19]; the results of our inhibition studies show that the efficacy of these compounds is effectively predicted by this model. The drug ciprofloxacin, which did not match the present pharmacophoric model, was proven here to have only a marginal effect on DHOdehase activity. The drug toltrazuril, which was shown to match the model to a lesser degree, exhibited a moderate inhibitory effect on the oxidation of dihydroorotate by rat liver mitochondria. The pharmacophoric model supports the concept that an enzyme will accept a molecule not only if it recognizes a certain number of key structural features but also if there are certain spatial arrangements of functional groups which fit in a spatially restricted area of the protein. The model proposed in this work is in partial agreement with studies published by Kuo *et al.* [12], who proposed an alignment of brequinar with HR325 based upon three pharmacophoric points. The superposition shown in Fig. 3 is based on our six-point model. Two lipophilic binding regions are represented by the biphenyl group and the benzo moiety of the quinoline ring in brequinar and the 4-substituted phenyl ring and the terminal methyl group in A77-1726. The hydrogen bond acceptors are one of the carboxylic oxygens and the quinolinic nitrogen in brequinar, and the carbonyl and the enolic oxygen in A77-1726.

The present study clearly demonstrated that extrapolation of findings from a model as the sole guide to predict the intracellular effects upon membrane-bound enzymes could be problematic. Our experiments revealed some interference of the agents (A77-1726, MNA715, MNA279, and brequinar at higher concentrations; HR325 below 10 μM) with two other mitochondrial redox systems: complex I and complex II of the respiratory chain. Because these as well as DHOdehase catalytically interact with ubiquinone in the inner mitochondrial membrane, the area of drug-enzyme interactions could be this contact site, preventing the transfer of electrons from the flavin redox center. All three activities involve integral flavin moieties as redox centers (FMN or FAD); however, only the NADH and succinate oxidase systems involve known Fe-S centers that mediate the flavin oxidation and quinone reduction, whereas such clusters are not known for DHOdehase [18, 34]. Thus, the special efficacy of the isoxazol and cinchoninic acid derivatives for DHOdehase might derive from the difference in flavin-quinone linkage. The diazine derivative toltrazuril exhibited a comparable efficacy for NADH and dihydroorotate oxidation, which both imply the redox cofactor FMN, but the same drug concentration (10 μM, Table 2) did not impair succinate oxidation by complex II, which contains covalently bound FAD. The structural difference between the FMN and FAD molecules could be more significant for the mode of diazine action than the quinone dependence of the three enzyme systems. Since no amino acid sequence homology of DHOdehase and protein subunits of complex I and complex II have been determined so far, the common inhibitory principle detected here—especially of the isoxazol compound HR325 with three redox activities—could be a useful probe to elucidate the structure of these proteins in the future.

The high concentration of the isoxazol and cinchoninic acid derivatives used in the present assays to discriminate their efficacy with three different redox processes might not be relevant to clinical conditions [5, 20]. However, putative disturbance of cell energetics and subsequent impediment in ATP-dependent enzymatic reactions should be taken into account when testing high drug concentrations on cell functions *in vitro* [4, 21].

On the basis of the present work and because rodent and human DHOdehase have become only recently available to us in sufficient purity and quantity [18, 34], extensive kinetic studies are in progress to establish and differentiate the individual profiles of inhibition by the agents used here. Having kinetic data at hand, the superposition rule given by our model could be used to derive three-dimensional quantitative structure-activity relationships (3D-QUASAR). This will open the door for a rational design of novel inhibitors of dihydroorotate dehydrogenase with high specificity.

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