Redoxal as a new leadstructure for dihydroorotate dehydrogenase inhibitors: a kinetic study of the inhibition mechanism

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Abstract Mitochondrial dihydroorotate dehydrogenase (DHOdehase; EC 1.3.99.11) is a target of anti-proliferative, immunosuppressive and anti-parasitic agents. Here, redoxal, (2,2′-[3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)diimino]bis-benzoic acid, was studied with isolated mitochondria and the purified recombinant human and rat enzyme to find out the mode of kinetic interaction with this target. Its pattern of enzyme inhibition was different from that of cinchoninic, isoxazol and naphthoquinone derivatives and was of a non-competitive type for the human (K_{ic} = 402 nM; K_{iu} = 506 nM) and the rat enzyme (K_{ic} = 116 nM; K_{iu} = 208 nM). The characteristic species-related inhibition of DHOdehase found with other compounds was less expressed with redoxal. In human and rat mitochondria, redoxal did not inhibit NADH-induced respiration, its effect on succinate-induced respiration was marginal. This was in contrast to the sound effect of atovaquone and dichloroallyl-lawsone, studied here for comparison. In human mitochondria, the IC_{50} value for the inhibition of succinate-induced respiration by atovaquone was 6.1 μM and 27.4 μM for the DHO-induced respiration; for dichlorallyl-lawsone, the IC_{50} values were 14.1 μM and 0.23 μM.

Key words: Dihydroorotate dehydrogenase; Redoxal; 1,4-Naphthoquinone; Pyrimidine; Mitochondrion

1. Introduction

The elucidation of the biochemical defects responsible for certain clinical immunodeficiencies [1] and the importance of the de novo pyrimidine and purine nucleotide synthesis for lymphocyte activation and proliferation [2] has stimulated interest in enzymes of nucleotide metabolism as intracellular targets for the development of immunosuppressive agents. Isoxazol and cinchoninic acid derivatives, such as brequinar [3,4], leflunomide, MNA715, MNA279 [5–8], have been shown to be potent inhibitors of human dihydroorotate dehydrogenase (DHOdehase), the fourth enzyme of pyrimidine de novo synthesis. Leflunomide (ARAVA™) passed clinical trials and received approval for the treatment of rheumatoid arthritis [5], whereas atovaquone is on clinical application against the malaria parasite *Plasmodium falciparum* and *Pneumocystis carinii* causing opportunistic infections in immunosuppressed patients [9,10]. Lawsone derivatives, such as dichloroallyl-lawsone (DCL) [11] and triazine derivatives [8,12], were also shown to interfere with the oxidation of DHO in mammalian cells and parasites. The ability of some DHOdehase inhibitors to suppress virus replication, presumably by lowering the intracellular pyrimidine pools, has also been taken into strategies of therapy [13].

In mammals, the enzyme DHOdehase is located in the inner mitochondrial membrane and intimately connected to the respiratory chain with ubiquinone as the proximal electron acceptor and cytochrome *c* oxidase (EC 1.9.3.1) as the ultimate electron transfer system. Thereby, DHOdehase resembles NADH dehydrogenase (EC 1.6.5.3) and succinate dehydrogenase (EC 1.3.5.1) of the inner membrane. The other five enzymes involved in pyrimidine de novo biosynthesis are located in the cytosol of cells [14].

The compound redoxal, originally found by the computer algorithm COMPARE within the National Cancer Institute's drug screening programme, was examined for its effect on pyrimidine biosynthesis in MOLT-4 human leukemia cells [15]. Since the interaction of redoxal with its putative target DHOdehase was not demonstrated with the purified enzyme species in vitro, we initiated the present study in order to elucidate the inhibition mechanism and to underline its attractive leadstructure for the further development of anti-pyrimidine drugs.

Due to its low abundance and membrane protein nature, sufficiently pure DHOdehase for inhibitor studies was very difficult to obtain until the recent availability of recombinant rat and human DHOdehase purified from insect cells used for heterologous overexpression [7,16]. The effect of redoxal on the purified rat enzyme was also evaluated here, because rodents are taken as animal models of human diseases or metabolism for in vivo testing of biologically active compounds. In addition, we studied the effects of redoxal on the respiratory chain activity in rat and human mitochondria in comparison to the 1,4-naphthoquinone analogs DCL and atovaquone (Fig. 1), this in order to differentiate their putative effect on electron transport processes different from the DHOdehase-catalysed oxidation of DHO to orotate.

2. Materials and methods

2.1. Material

Unless otherwise stated, all chemicals were from Boehringer Mannheim, Serva, Merck or Sigma, at the purest grade available. The inhibitors studied were obtained from: redoxal ((2,2′-[3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)diimino]bis-benzoic acid, NSC-73735) and DCL (2-hydroxy-3-(3,3-dichloroallyl)-1,4-naphthoquinone, NSC-

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Abbreviations: DHOdehase, dihydroorotate dehydrogenase (EC 1.3.99.11); DCL, dichloroallyl-lawsone; Q_D, decylubiquinone; DHO, L-dihydroorotate

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126771), NIH, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment Bethesda, USA; atovaquone (*trans*-2-(4-(chlorophenyl)cyclohexyl)-3-hydroxy-1,4-naphthoquinone), The Wellcome Foundation, Dartford, Kent, UK. Recombinant human and rat DHOdehase were gained and purified as described previously [7,16]. Rat and human liver mitochondria (human liver cells were obtained from routine histological protocol, Institute of Pathology, University of Marburg) were prepared according to standard protocols [7,16]. These organelles were 'broken' mitochondria, due to the lack of osmotic support for intactness of the membranes and as evidenced by high NADH oxidation [8]. These were taken here to evaluate the effect of drugs on the oxidation of DHO, NADH and succinate, without limitation on transport or uptake.

2.2. Enzyme assays

2.2.1. Oxygen consumption measurements. Mitochondria from the frozen stocks were transferred to a vessel connected to a Clarke-type electrode (Oxylab 1.81b System, Biolytik) and equilibrated for 2 min in the incubation medium (0.07 M saccharose, 0.2 M mannitol, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 4 mM HEPES pH 7.4). Protein content was around 2 mg/ml for rat and 3–6 mg/ml for human liver mitochondria. After addition of 5 mM succinate or 0.3 mM NADH, oxygen consumption was determined amperometrically: the inhibitors were added 1 min later. For control, the inactivation of the flux of electrons was achieved upon addition of 40 μM antimycin. Stocks of redoxal and brequinar were dissolved in buffer; atovaquone and DCL in DMSO with further dilution in DMSO.

2.2.2. Spectrophotometric tests. With isolated mitochondria and 1 mM DHO as substrate, DHOdehase was measured by determination of the orotate produced in the supernatant of acid-precipitated samples at 280 nm [7]. Recombinant human and rat DHOdehase were obtained from baculovirus-infected insect cells by purification protocols described previously. The activity ratio between the recombinant enzyme and the endogenous insect DHOdehase as determined in non-infected cells was 240:1 and 260:1, respectively [7,16]. Co-purification of the insect enzyme should have been negligible, because of the huge excess of recombinant enzyme and because the binding to the cationic exchange chromatography is expected to be different for the insect enzyme. There are only 51% of amino acid residues similar between the human and *Drosophila melanogaster* DHOdehase. Enzyme assays were done at 30°C according to previously described procedures using DHO and decylubiquinone (Q_D) as substrates and 2,6-dichlorophenol-indophenol as chromogene acceptor [7,16].

2.3. Kinetic analysis

Recent evaluation of kinetic constants for the recombinant DHOdehases under study gave the following values: $K_m = 9.4$ μM (DHO), $K_m = 13.7$ μM (Q_D) for the human enzyme [7]. $K_m = 11.5$ μM (DHO), $K_m = 5.9$ μM (Q_D) for the rat enzyme [17]. The half-maximal degree of inhibition (IC_{50}) for each inhibitor and the competitive (K_{ic}) and the uncompetitive inhibition constant (K_{iu}) were determined using the equations and programmes as described previously [17,18].

3. Results and discussion

3.1. Determination of kinetic constants

The present study consolidated the substantial interference of the compound redoxal with the oxidation of DHO to orotate in mammalian species. Here, the formal type of inhibition of redoxal was determined with the purified recombinant human and rat DHOdehase. For both species, a non-competitive type of inhibition was deduced from enzyme kinetics for both substrates, DHO and ubiquinone (Fig. 2). The inhibition constants are given in Table 1; those for the human enzyme were in the range described by Cleaveland et al. [15] who deduced the $K_i = 330$ nM from Dixon plot analysis of the DHOdehase inhibition in mitochondria of human MOLT-4 cells. The inhibition constants of redoxal for the rat enzyme were found to be 2–3-fold lower than for the human enzyme (Table 1). Since for the quinone co-substrate the K_{ic} value of 116 nM was found to be only half of the K_{iu} value (208 nM), the inhibition of the rat enzyme by redoxal should be classified as a 'mixed type' rather than a pure non-competitive type of inhibition [19]. Such a mixed type of inhibition could indicate that the binding of a compound involved both, the co-substrate (quinone) binding site and the catalysis [20]. In the case of rat DHOdehase, the inhibition pattern of redoxal resembled that of brequinar. By our previous work on brequinar and the rat enzyme [17], the K_{ic} value (Q_D) of 42 nM was calculated to be about 10-fold smaller than the K_{iu}.

As for the human DHOdehase, brequinar was shown to be a slow binding inhibitor with an overall inhibition constant of 1.8 nM. Its high affinity and characteristic interaction with the target enzyme were proposed to be the reason for its considerable side-effects and narrow therapeutic window with patients [17,21]. Since the inhibition profile and inhibition con-

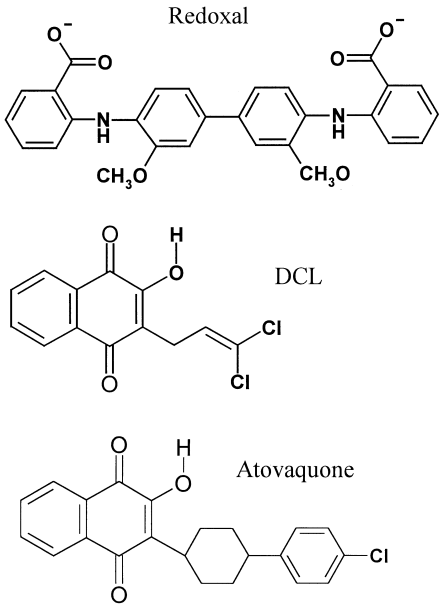

Fig. 1. Structures of redoxal, dichlorallyl-lawsone and atovaquone.

Table 1
Type of inhibition of DHOdehase by redoxal

Species	Variable substrate	Constant substrate	Type of inhibition	K_{ic} (nM)	K_{iu} (nM)
Human	DHO	Q_D	Non-competitive	487 ± 106	507 ± 39
	Q_D	DHO	Non-competitive	402 ± 83	506 ± 29
Rat	DHO	Q_D	Non-competitive	216 ± 40	233 ± 16
	Q_D	DHO	Non-competitive 'mixed type'	116 ± 22	208 ± 13

Inhibitor constants were determined by measuring the initial velocities of the DHOdehase-catalysed reaction in the presence or absence of the inhibitor. The velocities were determined either with varying concentrations of DHO (1.6–1000 μM) and 100 mM Q_D or with varying concentrations of Q_D (1.6–1000 μM) and 1 mM DHO. Inhibitor constants were derived from best fits of all data and are given ± asymptotic S.E.M.

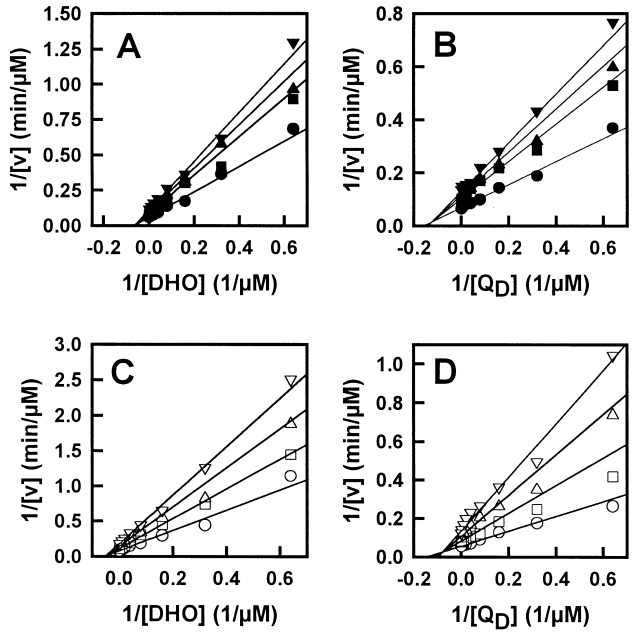

Fig. 2. Inhibition of human and rat DHOdehase by redoxal. DHOdehase activity was determined with the chromogen reduction assay. Initial velocities of human (A) and rat (C) DHOdehase as a function of one substrate concentration (DHO) at fixed concentrations of the second substrate (100 μM Q_D). Initial velocities of human (B) and rat (D) DHOdehase as a function of one substrate concentration (Q_D) at fixed concentrations of the second substrate (1 mM DHO). Double reciprocal plots of the data and fits (solid lines) are shown to demonstrate the type of inhibition. (A+B): ●, without inhibitor; ■, 250 nM redoxal; ▲, 350 nM redoxal; ▼, 450 nM redoxal. (C+D): ○, without inhibitor; □, 100 nM redoxal; △, 200 nM redoxal; ▽, 300 nM redoxal.

stants of redoxal were shown here to be quite different from that of brequinar, we would expect this new compound, or derivatives, to be of advantage for a depression of the DHOdehase activity in man. We, therefore, join the call of Cleavland et al. [15] that this novel anti-pyrimidine drug should merit consideration for development towards clinical trials, as soon as possible.

With respect to the substrate DHO, redoxal was found to be a non-competitive inhibitor of human DHOdehase (Table 1); this was in contrast to the isoxazol derivative leflunomide (active metabolite A771726, 2-hydroxyethylidene-cyanoacetic acid 4-trifluoromethyl anilide), which revealed an uncompetitive type of inhibition with the human enzyme [7,17]. Its inhibition constant was twice that of redoxal, whereas the constant was 10 times smaller than that of redoxal when the drugs were tested with the rat enzyme [16]. A considerable difference of inhibition pattern between the compound redoxal and DCL and atovaquone, respectively, could be deduced from the present study. By our recent work, both 1,4-naphthoquinone derivatives were shown to be competitive inhibitors of the purified rat and human DHOdehase with respect to the co-substrate Q_D [18]. DCL, with a K_{ic} of 9.8 nM for the human enzyme, turned out to act as a slow binding inhibitor of the rat enzyme with an overall inhibition constant of 0.77 nM; atovaquone revealed a $K_{ic} = 2.7$ μM for the human and a $K_{ic} = 60$ nM for the rat enzyme, respectively [18].

3.2. Effect of redoxal, DCL and atovaquone on respiratory chain activities

Since DCL and atovaquone were reported to be inhibitors of the mitochondrial respiratory chain in mammalian cells and parasites [11,22,23], these compounds and redoxal were tested here with mitochondria isolated from human and rat liver. Table 2 gives the results from oxygen consumption measurement with NADH, succinate and DHO as substrate. The IC_{50} values for atovaquone and DCL were lower with the mitochondrially bound rat DHOdehase than with the human enzyme. The same tendency was observed with the purified recombinant enzymes [18]. The IC_{50} values for redoxal clearly showed it to be a specific inhibitor of human (430 nM) and rat (910 nM) DHOdehase with fairly no effect on the ubiquinone dependent succinate and NADH dehydrogenase activities. In contrast, a considerable inhibition of the succinate oxidation by atovaquone and DCL can be deduced from the IC_{50} value, 6 μM and 14 μM with human mitochondria and 1 μM and 17 μM with rat mitochondria, respectively (Table 2). A weak effect of atovaquone and DCL on the NADH oxidation was detected. The IC_{50} values for atovaquone were in the same range as those reported by Fry and Pudney [23] with rat liver mitochondria. With human mitochondria, however, atovaquone proved to be a less potent inhibitor of DHOdehase than of succinate dehydrogenase. This would mean that in human, who is the host organism for malaria parasites, atovaquone would affect the respiratory chain and citric acid cycle to a greater extend than pyrimidine biosynthesis; whereas in the parasite *P. falciparum*, atovaquone seemed to interfere with pyrimidine de novo synthesis to a greater extend than with the electron transport. Our present findings on

Table 2
IC_{50} values for the inhibition of DHO, succinate and NADH oxidation in human and rat liver mitochondria

Species	Inhibitor	Substrate					
		DHO		Succinate		NADH	
		IC_{50} (μM)	(*n*)	IC_{50} (μM)	(*n*)	IC_{50} (μM)	(*n*)
Human	Atovaquone	27.4	(2)	6.1	(2)	71.5%[a]	(2)
Mitochondria	DCL	0.23	(2)	14.1	(2)	73%[a]	(2)
	Redoxal	0.43 ± 0.2	(4)	102 ± 19%[a]	(3)	96 ± 12%[a]	(3)
Rat	Atovaquone	0.79 ± 0.28	(3)	1.2 ± 0.4	(3)	3.2 ± 1.7	(3)
Mitochondria	DCL	0.013 ± 0.0002	(3)	17.5 ± 3.2	(3)	30.5 ± 8.9	(3)
	Redoxal	0.91 ± 0.23	(3)	79 ± 17%[a]	(3)	98 ± 8%[a]	(3)

Dose response curves for inhibition were obtained by varying the drug concentration from 1 nM up to 50 μM. The concentration to give half-maximal inhibition (IC_{50}) was determined by fitting the equation $v = V/(1+[I]/[IC_{50}])$ to the initial velocities. Results are means of *n* determinations and are given ± S.D.

[a]An IC_{50} value could not be determined within the tested range of inhibitor concentrations, therefore, the activity at the highest tested inhibitor concentration of 50 μM is given in % of the uninhibited reaction (100%).

the different degree of inhibition of NADH, succinate and DHO oxidation in mitochondria by redoxal underlined the non-competitive type of inhibition of purified DHOdehase by this compound and the competition for the DHOdehase quinone binding site by DCL and atovaquone.

The IC_{50} value for the inhibition of DHO oxidation (Table 2) by redoxal in rat mitochondria was higher than in human mitochondria. This seems to be in contrast to the inhibition constants determined with the purified enzymes (Table 1). In view of the relation between IC_{50} values and the inhibition constants, the IC_{50} was expected to be higher than K_{ic} in the case of partly competitive or mixed type inhibition ($K_{ic} = IC_{50}/(1+[A]/K_m)$) [24]. Only in the case of pure non-competitive inhibition, the relation $K_{iu} = K_{ic} = IC_{50}$ holds, as was shown here by enzyme kinetics with human DHOdehase in the presence of redoxal (487, 507 and 430 nM, Tables 1 and 2).

3.3. Concluding remarks

The pattern of inhibition kinetics displayed with the human DHOdehase and redoxal differs from that of all other inhibitors investigated in detail so far. In addition, the distinct species-related efficacy of inhibition of the human and rodent enzyme observed with isoxazol, cinchoninic acid and naphtoquinone derivatives [17,18] seemed to be less expressed with redoxal. Therefore, we conclude that the binding of redoxal may be divergent from that of leflunomide, brequinar, atovaquone and DCL. In contrast to DCL and atovaquone, redoxal did not interfere with respiratory chain activities. Since slow binding inhibition was not detected with the human nor with the rat DHOdehase, the toxic potential could be expected to be lower than that of brequinar and DCL. Therefore, we propose to follow redoxal as a leadstructure for the development of a new class of pharmacological drugs with a high specificity for DHOdehase and a high efficacy for lowering the pyrimidine nucleotide pools in mammalian cells.

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