Biochemical Pharmacology, Vol. 56, pp. 1259–1264, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00145-2

ELSEVIER

SHORT COMMUNICATION

Species-related Inhibition of Human and Rat Dihydroorotate Dehydrogenase by Immunosuppressive Isoxazol and Cinchoninic Acid Derivatives

*Wolfgang Knecht and Monika Löffler**

INSTITUTE FOR PHYSIOLOGICAL CHEMISTRY, SCHOOL OF MEDICINE, PHILIPPS-UNIVERSITY, MARBURG, GERMANY

ABSTRACT. The isoxazol leflunomide (N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide) and its active metabolite A77-1726 (N-(4-trifluoromethyl)-phenyl-2-cyano-3-hydroxy-crotonic acidamide) are promising disease-modifying antirheumatic drugs now in clinical trials. The malononitrilamides MNA279 (2-cyano-3-cyclopropyl-3-oxo-(4-cyanophenyl)propionamide) MNA715(N-(4-trifluoromethyl)-phenyl-2-cyano-3-hydroxy-hept-2-en-6-in-carboxylic acidamide) and HR325 (1(3-methyl-4-trifluoromethylphenyl-carbamoyl)-2-cyclopropyl-2-oxo-propionitrile) were shown to block rejection after allograft and xenograft transplantation in animals. Brequinar and other cinchoninic acid derivatives have also been evaluated as immuno-suppressive agents. A77-1726, HR325 and brequinar have been shown to have strong inhibitory effects on mitochondrial dihydroorotate dehydrogenase [EC 1.3.99.11], the fourth enzyme of pyrimidine *de novo* synthesis, with concomitant reduction of pyrimidine nucleotide pools. Pyrimidine nucleotides are essential for normal immune cell functions. Because most investigations had been carried out with cells, cell homogenates or mitochondrial fractions, it was the rationale of the present study to differentiate, under standardized conditions, the effect of leflunomide, A77-1726, MNA279, MNA715, HR 325 and brequinar on the recombinant rat and human enzymes, which were purified in our laboratory. Whereas leflunomide was a relatively weak inhibitor of the rat (IC_{50} = 6.3 μM) and human (IC_{50} = 98 μM) dihydroorotate dehydrogenase, the influence of A77-1726, MNA 279, MNA715 and HR325 was of comparable efficacy for either the rat (range of IC_{50}, 19–53 nM) or the human enzyme (range of IC_{50}, 0.5–2.3 μM). From the IC_{50} values, it was deduced that brequinar was a more potent inhibitor of the human dihydroorotate dehydrogenase activity (IC_{50} = 10 nM) than of the rat enzyme (IC_{50} = 367 nM). The rat enzyme was influenced by all isoxazol derivatives to a greater extent (IC_{50} = 19 nM A77-1726) than the human enzyme (IC_{50} = 1.1 μM A77-1726). These results may provide a plausible explanation for the findings of other laboratories with cultured cell lines and lymphocytes: in comparison to cells derived from human tissues, rat and other rodent cells were more susceptible to the isoxazol derivatives and less susceptible to brequinar. Our detailed kinetic investigations of the bisubstrate reaction catalyzed by rat dihydroorotate dehydrogenase revealed a noncompetitive type of inhibition by A77-1726 with respect to the substrate dihydroorotate and the cosubstrates ubiquinone or decylubiquinone. For brequinar, the inhibition was noncompetitive with respect to the substrate dihydroorotate, whereas with the quinone it was found to follow the "mixed typed" inhibition. In addition, brequinar acted as a "slow-binding" inhibitor of the human dihydroorotate dehydrogenase, a feature that might be of consequence for the reversibility of the reaction with the target. BIOCHEM PHARMACOL **56**;9:1259–1264, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cinchoninic acid; dihydroorotate dehydrogenase; human; isoxazol; kinetics; rat

Rheumatoid arthritis is an autoimmune disease characterized by severe inflammation of the joints, resulting in destruction of cartilage, bone, and tendon. Leflunomide,† an isoxazol derivative, is a novel immunosuppressive that has demonstrated efficacy and safety in the treatment of patients with active arthritis [1, 2] and that is presently in Phase III studies. Other isoxazol derivatives, the malononitrilamides MNA279 and MNA715, have been found to block rejection after allograft and xenograft transplantation in animals [3, 4]. Because *in vivo* leflunomide is rapidly processed to the active metabolite A77-1726, most *in vitro* studies have been performed using this formula. A77-1726 and HR325 were the first derivatives of this class for which a strong binding to the DHOdehase (EC 1.3.99.11), the fourth enzyme of pyrimidine *de novo* synthesis, was proven by radiolabeling studies [5]. Pyrimidine nucleotides serve essential functions in nucleic acid metabolism and the activation mechanism of sugars for glycosylation of proteins

* Corresponding author: Dr. Monika Löffler, Institute for Physiological Chemistry, Karl-von-Frisch-Strasse 1, D-35033 Marburg, Germany. Tel. +49-6421-285022; FAX +49-6421-286957; E-mail: loeffler@mailer.uni-marburg.de.

† *Abbreviations:* A77-1726, N-(4-trifluoromethyl)-phenyl-2-cyano-3-hydroxy-crotonic acidamide; DCIP, 2,6-dichlorophenolindophenol; DHOdehase, dihydroorotate dehydrogenase; FMN, flavinmononucleotide; HR325, 1(3-methyl-4-trifluoromethylphenyl-carbamoyl)-2-cyclopropyl-2-oxo-propionitrile; K_{ic}, competitive inhibition constant; K_{iu}, uncompetitive inhibition constant; leflunomide, N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide; MNA279, 2-cyano-3-cyclopropyl-3-oxo-(4-cyanophenyl)propionamide; MNA715, N-(4-trifluoromethyl)-phenyl-2-cyano-3-hydroxy-hept-2-en-6-in-carboxylic acidamide, Q_{10}, ubiquinone.

Received 7 November 1997; accepted 1 April 1998.

and lipids. By extensive work on various cell lines, the antipyrimidine effect of A77-1726 was demonstrated in various laboratories [6–8]. By kinetic studies using recombinant versions of the truncated human DHOdehase *in vitro*, characteristic features of the inhibitor A77-1726 were shown [9, 10]. The cinchoninic acid derivative brequinar (quinoline carboxylic acid [11]), which originally was discovered as a cytostatic agent with a strong effect on DHOdehase, is currently being evaluated for its effectiveness as an immunosuppressive agent for preventing graft rejection [12, 13]. From experiments with established cell lines, both A77-1726 and brequinar were found to affect cells from various animal species, although to different extents. Human cancer cell lines were found to be much more sensitive to the antiproliferative effect of brequinar than rat cells [14], and immune cell-specific reactions were observed to be more disrupted by low concentrations of A77-1726 in rodent than human lymphocytes [15]. In order to discover an underlying molecular mechanism for these observations of a species-related efficacy of the drugs, it was the rationale of the present experiments to study the effect of A77-1726 and brequinar on rodent DHOdehase by means of enzyme kinetics. The recent availability of purified recombinant rat and human DHOdehase [10, 16], which were proved to contain the redox cofactor FMN, allowed us to compare the inhibitory features of the four isoxazol derivatives (A77-1726, MNA279, MNA715 and HR325) and the cinchoninic acid derivative brequinar under identical assay conditions *in vitro*.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were from Boehringer Mannheim, Serva, Merck or Sigma, at the purest grade available. Leflunomide, A77-1726, MNA279, MNA715 and HR325 were obtained from Hoechst Marion Roussel Deutschland GmbH. Brequinar Sodium (NSC 368390), 6-fluoro-2-(2′-fluoro-1, 1′-biphenyl-4yl)-3-methyl-4-quinoline carboxylic acid sodium was obtained from DuPont Pharma GmbH. L-Dihydroorotate, Q_D, Q_{10} and DCIP were from Sigma.

The recombinant truncated human DHOdehase (43 kDa; EMBL sequence data bank Accession No. M94065) and the complete rat DHOdehase(42.7 kDa; EMBL sequence data bank Accession No. X80778) were purified as described previously by Knecht *et al.* [10, 16]. For the DHOdehase protein, a molecular mass of 43-44 kDa was deduced from SDS-PAGE of rat liver mitochondria, in correction of previous reports [17, 18], and 43 kDa for the enzyme of human liver mitochondria [10, 16]. At the N-terminus of the rat enzyme, a typical but short mitochondrial targeting sequence of 10 amino acids was identified; the N-terminal 11 amino acids that are known for the human enzyme include the targeting information for the import into the mitochondria as well. Because this sequence (Xaa_n-Lys-Leu-Pro-Trp-Arg-His-Leu-Gln-Lys-Arg-Ala-) is longer than that of the rat enzyme (Met-Ala-Trp-Arg-Gln-Leu-Arg-Lys-Arg-Ala-), it can be assumed that very few amino acids are lacking at the N-terminus of the human enzyme.

Enzyme Assay

Enzyme assays with the recombinant DHOdehase after purification were performed at 30° according to previously described procedures [10, 16, 17]. The oxidation of the substrate dihydroorotate and the reduction of the cosubstrate quinone was coupled to the reduction of the chromogen DCIP. The reaction mixture contained 0.1 mM Q_D or 0.1 mM Q_{10}, 1 mM L-dihydroorotate, 0.06 mM DCIP, 0.1% Triton X-100 in 50 mM Tris-HCl buffer, 150 mM KCl, pH 8.0. The reaction was started by addition of the enzyme. The loss in absorbance of the blue DCIP was monitored at 600 nm; $\epsilon = 18.800\ l\ mol^{-1}\ cm^{-1}$. The enzyme activity in control assays without Q_D or Q_{10} was approximately 1% maximum enzyme activity; this amount was subtracted from the activity values measured. No dye reduction was detected if the control assay was performed in the presence of the enzyme and Q_D or Q_{10} but in the absence of dihydroorotate.

Stock solutions of 10 mM leflunomide, HR325, MNA715 and MNA279 were prepared in dimethylsulfoxide with further dilutions in the buffer taken for the assays. A77-1726 and brequinar were dissolved in buffer. All solutions were fresh preparations. Dimethylsulfoxide at the stated dilutions was found not to interfere with DHOdehase activity.

Kinetic Analysis

Recent evaluation of kinetic constants for the DHOdehases under study gave the following values: K_m for dihydroorotate = 4 μM and K_m for Q_{10} = 9.9 μM; K_m for dihydroorotate = 9.4 μM and for Q_D = 13.7 μM (human enzyme [10]); K_m for dihydroorotate = 6.4 μM and for Q_{10} = 9.9 μM; K_m for dihydroorotate = 11.5 μM and for Q_D = 5.9 μM (rat enzyme [16]). To determine the inhibitory potency of the agents, the initial velocity of the DHOdehase reaction was measured at saturating substrate concentrations, 1 mM dihydroorotate and 100 μM Q_D and varying concentrations of the drugs (1 nM through 100 μM). The equation was fitted to the initial velocities:

$$v = V/\{1 + [I]/IC_{50}\} \quad (1)$$

([I] is the inhibitor concentration) in order to find the concentration causing 50% inhibition of the enzyme activity (IC_{50}).

To identify the type of inhibition and to estimate the values of the inhibition constant, the following experiments were designed. Three fixed inhibitor concentrations were chosen for three sets of assays. The initial velocities of each were determined either with varying concentrations of dihydroorotate and 100 μM Q_D or 100 μM Q_{10}, or with

TABLE 1. IC_{50} values describing the effect of isoxazol derivatives and brequinar on human and rat DHOdehase

Compound	Human DHODH IC_{50} (μM)	n	Rat DHODH IC_{50} (nM)	n
Leflunomide	97.9 ± 34	3	6300 ± 3300	3
A77 1726	1.08 ± 0.1	5	19 ± 7	3
MNA 279	2.25 ± 0.4	6	25.8 ± 3.5	3
MNA 715	0.46 ± 0.17	6	52.5 ± 6.7	3
HR 325	1.11	2	20.1	2
Brequinar	$10.3 \times 10^{-3} \pm 0.9 \times 10^{-3}$ (v_f)	3	366.9 ± 100	3

Human and rat DHOdehase activity was determined with the chromogen reduction assay using DCIP, dihydroorotate and Q_D at saturating concentrations. Dose-response curves for inhibition of the two enzymes were obtained by varying the drug concentrations from 1 nM to 100 μM. The concentration of inhibitor required to achieve the half-maximal degree of inhibition (IC_{50}) was determined by fitting the equation $v = V/\{1 + [I]/IC_{50}\}$ to initial velocites as explained under *Materials and Methods*. Results are means ± S.D. from the indicated number (n) of experiments. (v_f): Brequinar was found to act as slow-binding inhibitor; therefore steady-state velocities were used to determine IC_{50}.

varying concentrations of Q_D or Q_{10} and 1 mM dihydroorotate. The equation was fitted to the data:

$$v = V \times [A]/(K_m + [A]) \quad (2)$$

([A] is the substrate concentration). It is obvious from the values presented in Table 1 that the rat enzyme was affected to a greater extent than the human DHOdehase by all isoxazol derivatives. The IC_{50} values of A77-1726 and HR325 obtained with the human enzyme were 20-fold those obtained with the rat DHOdehase, with the greatest difference observed in the case of the MNA715. In contrast, the cinchoninic acid brequinar was found to be a more potent inhibitor of the human enzyme, the IC_{50} obtained with the rat enzyme being 30- to 40-fold that observed with the human enzyme.

Rodents, preferentially mice but also rats, and rat or mice cell lines are important tools in evaluating properties of new drugs prior to studies evaluating drug effects in human beings. This prompted the present detailed kinetic study with recombinant rat DHOdehase to be performed, so as to compare it with the human enzyme. Because IC_{50} values give the net effect of inhibitors on enzyme activity and depend on assay conditions and type of inhibition, the ultimate criterion by which the effectiveness of a compound is judged, i.e., the true inhibition constant for the enzyme, was determined [21]. In previous work with the recombinant human DHOdehase, we found A77-1726 to be an uncompetitive inhibitor with respect to dihydroorotate (K_{iu} = 0.94 μM) and a noncompetitive inhibitor with respect to Q_D (K_{ic} = 1.09 μM, K_{iu} = 1.05 μM) [10]. This convenient quinone has often been used for assays of dehydrogenases *in vitro*. Essentially the same results were found when the experiments were now repeated with Q_{10}, the most abundant ubiquinone in mammals: A77-1726 is an uncompetitive inhibitor of human DHOdehase with respect to dihydroorotate (K_{iu} = 1.3 ± 0.07 μM) and a noncompetitive inhibitor with respect to Q_{10} (K_{ic} = 1.5 ± 0.45 μM, K_{iu} = 1.2 ± 0.13 μM). Greene *et al.* [6] reported mixed-type kinetics with dihydroorotate and human mitochondria: the kinetic constants, K_{is} = 3.28 ± 2.45 μM; K_{ii} = 1.16 ± 0.35 μM, were close to ours assuming that $K_{is} = K_{ic}$ and $K_{ii} = K_{iu}$ [20]; $K_{ic} > K_{iu}$ may indicate a predominantly uncompetitive inhibition [19]. For the quinone binding of the human enzyme (a minus 30 amino acid truncated recombinant variant without detectable FMN as redoxcofactor [22]), a competitive type of inhibition for A77-1726 (K_i = 179 ± 19 nM) was observed by Davis *et al.* [9]. The same study revealed noncompetitive inhibition for dihydroorotate, whereas brequinar was reported to be a noncompetitive inhibitor for both the dihydroorotate and ubiquinone binding sites of the enzyme, with a K_i = 26 nM as obtained from Dixon plot [22].

Deviations of V and K_m values in comparison to the constants for the noninhibited enzymatic reaction were considered to determine whether the inhibition was competitive, uncompetitive or noncompetitive, respectively [19]. Once an inhibition pattern was established, the appropriate equations were fitted to the data as:

competitive inhibition: $v = V \times [A]/\{K_m \times (1 + [I]/K_{ic}) + [A]\}$

uncompetitive inhibition: $v = V \times [A]/\{K_m + (1 + [I]/K_{iu}) \times [A]\}$ or

noncompetitive inhibition: $v = V \times [A]/\{K_m \times (1 + [I]/K_{ic}) + (1 + [I]/K_{iu}) \times [A]\}$.

(K_{ic} is the competitive inhibition constant; K_{iu} is the uncompetitive inhibition constant [20]).

RESULTS AND DISCUSSION

The availability of purified DHOdehase from mitochondrial membranes of animal tissues for extensive *in vitro* studies was extremely limited until the successful overexpression and purification of the rat and human enzyme species to high specific activity [10, 16]. We previously reported that the catalytic properties of the two recombinant enzymes were very similar to those determined for the enzyme isolated from tissues; hence, we used the recombinant DHOdehases to compare the effect of isoxazol and

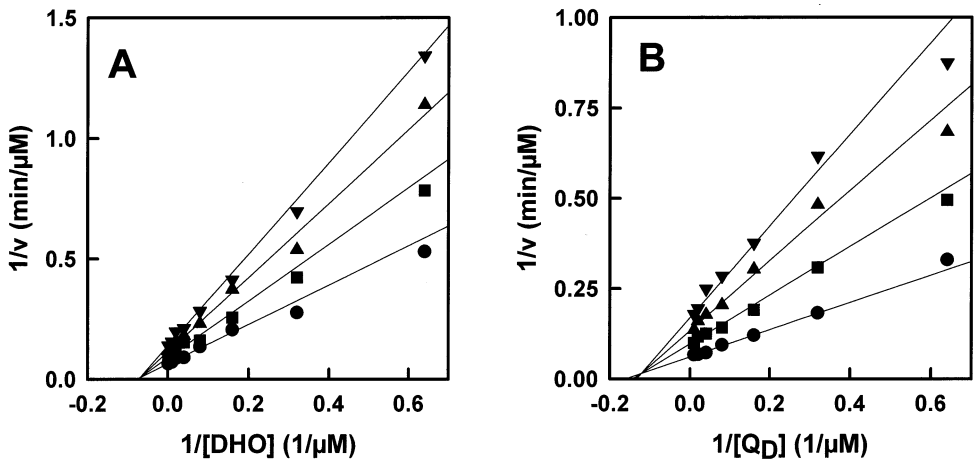

FIG. 1. Kinetic pattern for the reaction of rat DHOdehase with A77-1726 and Q_D as cosubstrate. DHOdehase activity was determined with the chromogen reduction assay using DCIP, Tris-HCl buffer, pH 8, at 30° and the substrate concentrations as indicated: (A) Initial velocities of DHOdehase as a function of one substrate concentration (dihydroorotate) at fixed concentrations of the second substrate (100 µM Q_D). Double reciprocal plot of the data to demonstrate the type of inhibition; (B) Initial velocites of DHOdehase as a function of one substrate concentration (Q_D) at fixed concentrations of the second substrate (1 mM dihydroorotate). Double reciprocal plot of the data demonstrate type of inhibition. (●) Without inhibitor; (■) 10 nM A77-1726; (▲) 20 nM A77-1726; (▼) 30 nM A77-1726. The solid lines represent the best fit of the equations given under *Materials and Methods* to the data.

cinchonionic acid derivatives on this target under standardized assay and laboratory conditions.

The IC_{50} value as a practical readout of the relative effects of different substances on enzyme activity under comparable conditions was obtained from dose-response plots [21]. The results of activity measurements of the human and rat DHOdehase in the presence of leflunomide, A77-1726, MNA279, MNA715, HR325, and brequinar are summarized in Table 1. As for the mother compound of isoxazolderivatives, leflunomide, a remarkably low potency was seen in comparison to the metabolite A77-1726 and its analogs (Table 1). This finding clearly shows that leflunomide with its intact isoxazol ring has to be converted by ring-opening to A77-1726 in order to become a potent inhibitor of the target DHOdehase. Quite a good inhibition of human DHOdehase by leflunomide (K_i = 4.6 µM) was reported by Greene *et al.* [6]; however, the authors could not rule out a conversion of leflunomide to A77-1726 through their tests with crude mitochondria preparations, and they noted a need to check their findings on purified DHOdehase.

We then performed comprehensive kinetic analyses of the recombinant rat enzyme as influenced by A77-1726. Because virtually the same profiles were obtained with the cosubstrate Q_D (Fig. 1) and Q_{10} (not shown), it was concluded that decylubiquinone can be used for all *in vitro* studies with DHOdehases. It is obvious from Table 2 that in both cases the inhibition constants of A77-1726 were smaller for the rat enzyme than for the human DHOdehase (see above). In addition, kinetic measurements revealed a change in the inhibition pattern of A77-1726 with respect to dihydroorotate from the uncompetitive type—as for the human enzyme—to the noncompetitive type with the rat DHOdehase. For comparison, the inhibition kinetics of the rat enzyme with brequinar gave the following results: with dihydroorotate as variable substrate, a clear noncompetitive type of inhibition could be deduced from comparable values of K_{ic} (308 ± 64 nM) and K_{iu} (289 ± 20 nM). A great difference in these values—K_{ic} (42 ± 7 nM) and K_{iu} (507 ± 39 nM) for the co-substrate Q_D here—has been classified as a "mixed-type" rather than a noncompetitive type of inhibition [19]. Mixed-type inhibition could indicate that the binding of an inhibitor involved both the substrate binding site and catalysis [23]. Therefore, a competition of brequinar with the quinone for the binding site in the rat DHOdehase could be deduced from the results.

TABLE 2. Kinetic analyses of rat DHOdehase inhibitor by A77-1726

Variable substrate	Constant substrate	Type of inhibition	K_{ic} (nM)	K_{iu} (nM)
DHO (1.56–1000 µM)	Q_D (100 µM)	Noncompetitive	22.6 ± 4.6	25.8 ± 1.8
DHO (1.56–1000 µM)	Q_{10} (100 µM)	Noncompetitive	11.4 ± 2.4	16.7 ± 1.1
Q_D (1.56–100 µM)	DHO (1 mM)	Noncompetitive	12.6 ± 1.9	16.8 ± 1.0
Q_{10} (1.56–100 µM)	DHO (1 mM)	Noncompetitive	13.1 ± 3.5	15.1 ± 2.1

Inhibitor constants for competitive inhibition (K_{ic}) and uncompetitive inhibition (K_{iu}) were determined by measuring the initial velocities of the DHOdehase-catalyzed reaction in the presence or absence of A77-1726. Three fixed inhibitor concentrations (10, 20 and 30 nM) were chosen for three sets of assays. The initial velocities of each were determined either with varying concentrations of dihydroorotate (DHO) and 100 µM decylubiquinone (Q_D) or 100 µM ubiquinone (Q_{10}), or with varying concentration of Q_D or Q_{10} and constant 1 mM dihydroorotate. Once an inhibition pattern was found, the appropriate equation, given under *Materials and Methods*, was fitted to all data. Inhibitor constants are best fit of all data ± asymptotic standard error.

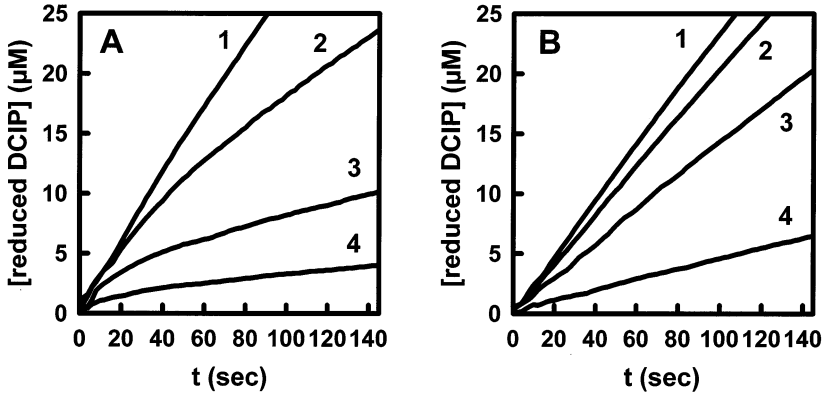

FIG. 2. Time-dependent inhibition of human DHOdehase by Brequinar and A77-1726. DHOdehase activity was determined with the chromogen reduction assay using DCIP, dihydroorotate and Q_D, Tris-HCl buffer, pH 8, at 30° and the inhibitor as indicated. The reaction was started by addition of the enzyme to the reaction mixture. (A) Brequinar at 0 nM (1); 10 nM (2); 31.6 nM (3); 100 nM (4); (B) A77-1726 at 0 μM (1); 0.1 μM (2); 1 μM (3); 10 μM (4).

Figure 2 shows the time-dependent inhibition of human DHOdehase activity by brequinar and A77-1726. The pattern of inactivation by brequinar (Fig. 2A) through the first 50 sec was different from that obtained with A77-1726 (Fig. 2B). This slow inactivation by Brequinar was interpreted as a characteristic feature of a "slow-binding inhibitor" (for review, see Morrison [24] and Szedlacsek and Duggleby [25]). Slow-binding kinetics have not been reported for the partial recombinant human enzyme [22]; however, it is not known whether the course of the activity assays had been monitored immediately upon addition of the enzyme as was done in our study. With the rat enzyme, brequinar did not reveal a slow-binding kinetic profile (data not shown), but rather one comparable to that obtained with the human enzyme and A77-1726 (Fig. 2B). The kinetic measurements with the isoxazol derivatives A77-1726, MNA715, MN279, and HR325 did not display profiles of a slow-binding character (data not shown). Evidence has accumulated that many slow-binding inhibitors mimic intermediates in enzyme catalysis; some of them have been thought to cause irreversible damage to the target [21, 26]. The subtle difference in the brequinar-target interaction disclosed by the present study with the rat and the human enzyme should be the subject of further studies, especially with DHOdehases from other species.

Our hypothesis was that differences among cells from different species in their susceptibility to the isoxazol and cinchoninic acid derivatives as described by different authors could be based on different affinities for the drugs by the enzyme DHOdehase. To date, the primarily discussed target of the cinchoninic acid and isoxazol derivatives in cells has been the inhibition of DHOdehase, the fourth enzyme in UMP biosynthesis [6, 7, 8, 18, 27]. This does not exclude the possibility that the compounds may interfere with other cell components as well, e.g. at higher concentrations [28]. The drugs were thought to mediate their effect on immune cells by decreasing pyrimidine nucleotide pools. It has been emphasized that pyrimidine nucleotides are essential for normal immune function [29]. The mitogen-induced T lymphocyte proliferation of rat cells was reported to be more sensitive to A77-1726 (IC_{50} = 0.086 μM) than of human cells (IC_{50} = 12.6 μM) [14], and the IC_{50} value for brequinar as evaluated with the mixed lymphocyte response assay was shown to be 0.391 μM (rat) and 0.014 μM (human) [27]. Similarly, experiments with the malononitrilamides on T and B cell responsiveness and proliferation, revealed a more prominent susceptibility of rat cells (IC_{50} = 0.5 μM MNA279; IC_{50} = 1 μM MNA715; IC_{50} = 0.1 μM A77-1726) in comparison to human cells (IC_{50} = 18.9 μM MNA279; IC_{50} = 5.6 μM MNA715 and IC_{50} = 4.7 μM A77-1726) [30]. A reversed efficacy of growth inhibition was found with brequinar, e.g. M5-melanoma: IC_{50} = 0.35 μM; 14C-squamous: IC_{50} = 0.19 μM; H35 rat hepatoma: IC_{50} = 2.53 μM [13]. Via this type of IC_{50} determination, the drugs were added to the cell medium and the effects of inhibition were measured indirectly by a readout of biological activity, e.g. influence on cell growth and proliferation, that is dependent on the activity of the target enzyme DHOdehase. From the IC_{50} values obtained, it can be concluded that the different susceptibility of the rat and human cells to the isoxazol or cinchoninic acid derivatives, respectively, correlates with an appropriate repression of DHOdehase activity as evaluated by the present kinetic study. Experiments with other classes of anti-DHOdehase compounds are in progress in this laboratory in an attempt to confirm our notion that kinetic parameters are a reliable tool to predict different grades of efficacy for cells from different animal species.

This study was supported by Deutsche Forschungsgemeinschaft, Graduierten-Kolleg Enzymchemie and by a research grant from the Hoechst-Marion-Roussel AG. The authors wish to thank Johannes Henseling for his expert technical assistance.

References

1. Bartlett RR, Dimitrijevic M, Mattar T, Zielinski T, Germann T, Rüde E, Thoenes GH, Küchle CCA, Schorlemmer HU, Bremer E, Finnegan A and Schleyerbach R, Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions to transplantation rejection. *Agents Actions* **32:** 10–21, 1991.
2. Mladenovic V, Domljan Z, Rozman B, Jajic I, Mihajlovic D, Dordevic J, Popovic M, Dimitrijevic M, Zivkovic M, Campion G, Musikic P, Loew-Friedrich I, Oed C, Seifert H and Strand V, Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results

of a randomized, placebo-controlled, phase II study. *Arthritis Rheum* **38:** 1595–1603, 1995.
3. Silva HT and Morris RE, Leflunomide and malonitrilamides. *Exp Opin Invest Drugs* **6:** 51–64, 1997.
4. Schorlemmer HU, Kurrle R and Bartlett RR, Malononitrilamides inhibit the development of various murine graft-versus-host diseases. *Transplant Proc* **28:** 3043–3047, 1996.
5. Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Moss D, Thomson TA, Spinella-Jaegle S, Morand P, Courtin O, Sautés C, Westwood R, Hercend T, Kuo EA and Ruth E, Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* **270:** 22467–22472, 1995.
6. Greene S, Watanabe K, Braatz-Trulson J and Lou L, Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent Leflunomide. *Biochem Pharmacol* **50:** 861–867, 1995.
7. Cao WW, Kao PN, Chao AC, Gardner P, Ng J and Morris RE, Mechanism of the antiproliferative action of leflunomide. A77-1726, the active metabolite of leflunomide, does not block T-cell receptor-mediated signal transduction but its antproliferative effects are antagonized by pyrimidine nucleosides. *J Heart Lung Transplant* **14:** 1016–1030, 1995.
8. Xu X, Williams JW, Gong H, Finnegan A and Chong AS, Two activities of the immunosuppressive metabolite of leflunomide, A77-1726. Inhibition of pyrimidine nucleotide synthesis and protein tyrosine phosphorylation. *Biochem Pharmacol* **52:** 527–534, 1996.
9. Davis JP, Cain GA, Pitts WJ, Magolda RL and Copeland RA, The immuno-suppressive metabolite of Leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. *Biochemistry* **35:** 1270–1273, 1996.
10. Knecht W, Bergjohann U, Gonski S, Kirschbaum B and Löffler M, Functional expression of a fragment of human dihydroorotate dehydrogenase by means of the baculovirus vector system, and kinetic investigation of the purified enzyme. *Eur J Biochem* **240:** 292–301, 1996.
11. Chen SF, Ruben RL and Dexter DL, Mechanism of action of the novel anticancer agent 6-fluoro-2-(2′-fluoro-1.1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt (NSC 368390): inhibition of *de novo* pyrimidine nucleotide biosynthesis. *Cancer Res* **46:** 5014–5019, 1986.
12. Makowka L, Sher LS and Cramer DV. The development of Brequinar as an immunosuppressive drug for transplantation. *Immunol Rev* **136:** 51–70, 1993.
13. Batt DG, Copeland RA, Dowling RL, Gardner TL, Jones EA, Orwat MJ, Pinto DJ, Pitts WJ, Magolda RL and Jaffee BD, Immunosuppressive structure-activity relationships of Brequinar and related cinchoninic acid derivatives. *Bioorg Med Chem Lett* **5:** 1549–1554, 1995.
14. Peters GJ, Laurensse E, de Kant E, Nadel JC and Pinedo HM, The relationship between dihydroorotic acid dehydrogenase and *in vitro* and *in vivo* cytostatic effects of brequinar sodium (DUP-785, NSC 368390). *Adv Exp Med Biol* **253B:** 375–382, 1989.
15. Cherwinski HM, McCarley D, Schatzman R, Devens B and Ransom JT, The immunosuppressant Leflunomide inhibits lymphocyte progression through cell cycle by a novel mechanism. *J Pharmacol Exp Ther* **272:** 460–468, 1995.
16. Knecht W, Altekruse D, Rotgeri A, Gonski S and Löffler M, Rat dihydroorotate dehydrogenase: isolation of the recombinant enzyme from mitochondria of insect cells. *Protein Expr Purif* **10:** 89–99, 1997.
17. Lakaschus G and Löffler M, Differential susceptibility of dihydroorotate dehydrogenase/oxidase to Brequinar Sodium (NSC 368390) *in vitro*. *Biochem Pharmacol* **43:** 1025–1030, 1992.
18. Forman HJ and Kennedy J, Purification of the primary dihydroorotate dehydrogenase (oxidase) from rat liver mitochondria. *Prep Biochem* **7:** 345–355, 1977.
19. Cornish-Bowden A, *Fundamentals of Enzyme Kinetics*, Portland Press, London, 1995.
20. Liébecg C, *IUBMB Biochemical Nomenclature and Related Documents*, Portland Press, London, 1992.
21. Copeland RA, *Enzymes*. VCH Publishers, New York, 1996.
22. Copeland R, Davis JP, Dowling RL, Lombardo D, Murphy KB and Patterson TA, Recombinant human dihydroorotate dehydrogenase: expression, purification, and characterization of a catalytically functional truncated enzyme. *Arch Biochem Biophys* **323:** 79–86, 1995.
23. Voet D and Voet JG, *Biochemistry*. John Wiley & Sons, Inc., New York, 1995.
24. Morrison JF, The slow-binding and slow tight-binding inhibition of enzyme-catalysed reactions. *TIBS* **7:** 102–105, 1982.
25. Szedlacsek SE and Duggleby RG, Kinetics of slow- and tight-binding inhibitors. *Methods Enzymol* **249:** 144–181, 1995.
26. Röhm KH, Butylmalonate is a transition state analogue for aminoacylase I. *FEBS Letters* **250:** 191–194, 1989.
27. Cherwinski HM, Byars N, Ballaron SJ, Nakano GM, Young JM and Ransom JT, Leflunomide interferes with pyrimidine nucleotide biosynthesis. *Inflamm Res* **44:** 317–322, 1995.
28. Jöckel J, Wendt B and Löffler M, Structural and functional comparison of agents interfering with dihydroorotate, succinate and NADH oxidation of rat liver mitochondria. *Biochem Pharmacol* **56:** 1053–1059, 1998.
29. Fairbanks LD, Bofill M, Ruckemann K and Simmonds HA, Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of *de novo* synthesis. *J Biol Chem* **270:** 29682–29689, 1995.
30. Kurrle R, Ruuth E, Lauffer L and Schorlemmer HU, Malononitrilamides inhibit T- and B-cell responsiveness in different species. *Transplant Proc* **29:** 1302–1303, 1997.