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INHIBITION OF DIHYDROOROTATE DEHYDROGENASE BY THE IMMUNOSUPPRESSIVE AGENT LEFLUNOMIDE

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Abstract—Leflunomide [HWA 486 or RS-34821, 5-methyl-*N*-(4-trifluoromethylphenyl)-4-isoxazole carboximide] is an immunosuppressive agent effective in the treatment of rheumatoid arthritis. In spite of its clinical potential, its mechanism of action has not been elucidated. Recent studies suggest that leflunomide may interfere with the metabolism of pyrimidine nucleotides. In our studies, the active metabolite of leflunomide, RS-61980 (A77 1726, 2-hydroxyethylidene-cyanoacetic acid-4-trifluoromethyl anilide), was cytostatic towards a human T-lymphoblastoma cell line (A3.01). The inhibition of growth could be overcome completely by uridine. The other nucleosides, cytidine, adenosine and guanosine, did not overcome the effect of the compound. Since uridine is a precursor for the salvage synthesis of UMP, we propose that RS-61980 may be inhibiting the *de novo* pathway of UMP synthesis. Using human cells, the six enzymes catalyzing *de novo* UMP biosynthesis were tested for their sensitivity towards RS-61980. Only one of the enzymes, dihydroortate dehydrogenase (DHODH, EC 1.3.3.1) was inhibited by RS-61980 with a K_i value of 2.7 ± 0.7 μM. The other five enzymes were not affected. The inhibition exhibited mixed-type kinetics towards both substrates, dihydroorotic acid and coenzyme Q. These results suggest that the molecular target of leflunomide action is DHODH. The immunomodulating activity may be related to the inhibition of UMP synthesis in proliferating lymphocytes.

Key words: leflunomide; pyrimidine nucleotide synthesis; *de novo* pathway; Brequinar; dihydroortate dehydrogenase; uridine

The enzymes in the *de novo* synthesis of purine and pyrimidine nucleotides are proven targets for immunosuppressive agents. Mycophenolate mofetil [RS-61443, 6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-2-(4-morpholinyl)ethyl ester)], a prodrug of mycophenolic acid, is efficacious in the treatment of transplant rejection and rheumatoid arthritis [1, 2]. Mycophenolic acid is a potent inhibitor of IMPDH† (EC 1.1.1.205), a key enzyme of *de novo* synthesis of purine nucleotides [for a review, see Ref. 3]. Brequinar Sodium™ [NSC 368 390, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt, Brequinar], an inhibitor of DHODH in the *de novo* synthesis of pyrimidine nucleotides, is effective in preventing graft rejection [for a review, see Ref. 4].

Leflunomide is a novel immunosuppressive agent that is effective towards several autoimmune diseases and rejection of transplants in animals (Fig. 1) [for reviews, see Refs. 5 and 6]. It effectively reduces inflammation in animal models of rheumatoid arthritis, such as adjuvant arthritis and proteoglycan-induced progressive polyarthritis [7, 8]. It is currently being investigated in clinical trials for the treatment of rheumatoid arthritis. Despite its promising potential as an immunomodulatory drug, its molecular target has not been elucidated clearly. It has been proposed that the mechanism of action is related to the inhibition of both activation and proliferation of T lymphocytes [5]. Several groups have reported that the levels of interleukin 2 (IL-2) production, IL-2 receptor expression, and tyrosine kinase activity are reduced by the drug [9–12]. However, the inhibition of these events was very weak, and the data are highly controversial, since they have not been entirely reproducible among different groups of investigators. More importantly, the doses required for the inhibition of these events were substantially higher than the doses needed for inhibition of growth. These results do not explain the *in vitro* and *in vivo* potency of leflunomide. It is unlikely that inhibition of these early events of lymphocyte activation, such as cytokine production, receptor up-regulation and tyrosine kinase stimulation, represents the main feature of the immunosuppressive action of leflunomide [12].

Leflunomide is a general cytostatic agent for a wide variety of animal cells. *In vivo*, it is metabolized rapidly and the primary active metabolite is RS-61980 (see Fig. 1 for structure) [13]. The drug action seems to be late-acting in the cell cycle, inhibiting the progression from the S to G_2/M phase. It inhibits DNA and RNA syntheses, and its potency is unchanged even when added to lymphocytes that are past the early stages of activation. Unlike mycophenolic acid, the action of leflunomide is not overcome by purine nucleotides. Similar to Brequinar, however, its effects can be overcome by either uridine or cytidine in human PBL.‡

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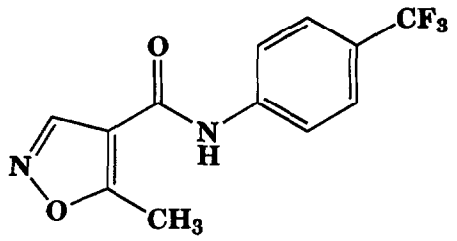
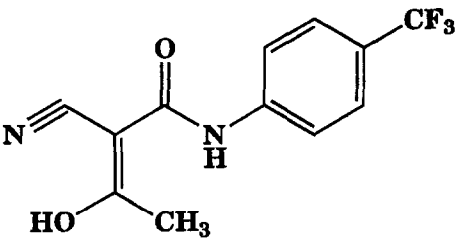
† Abbreviations: IMPDH, inosine 5′-monophosphate dehydrogenase; OMP, orotidine 5′-monophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; UMP, uridine 5′-monophosphate; DHODH, dihydroorotate dehydrogenase; CPS, carbamyl phosphate synthetase II; ATC, aspartate transcarbamylase; DHOase, dihydroorotase; OPRT, orotate phosphoribosyl transferase; OMPDC, orotidine 5′-monophosphate decarboxylase; and PBL, peripheral blood lymphocytes.

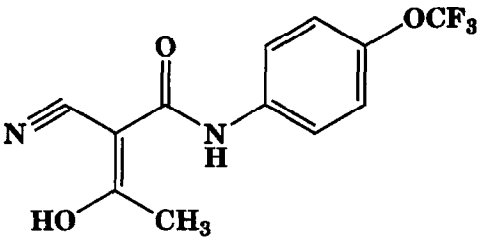
‡ Cherwinski H, Cohn RG, Cheung P, Webster DJ, Caufield JP, Young JM and Ransom JT, Manuscript submitted for publication. Cited with permission.



RS-34821
(leflunomide)



RS-61980



RS-56338

Fig. 1. Structures of leflunomide and related compounds.

A common feature of cytidine and uridine is that they can be salvaged to UMP. UMP is essential because it is the precursor of all pyrimidine nucleotides that a cell requires for growth and other functions. Cytidine can be readily converted to uridine by cytidine deaminase, and uridine can be salvaged directly to UMP by uridine/cytidine kinase. Alternatively, cytidine can be salvaged to CMP by the same kinase and then converted to UMP by cytidylate deaminase. Both of the above schemes result in the salvage synthesis of UMP from uridine or cytidine. Salvage synthesis is one of two pathways for UMP generation; the other pathway is *de novo* synthesis [for a review, see Ref. 14]. Since generation of UMP from uridine or cytidine bypasses the *de novo* pathway, the fact that the inhibitory effects of leflunomide can be overcome by uridine or cytidine suggests that the drug may affect the *de novo* synthesis of UMP. A depletion of the UMP level by inhibition of the *de novo* synthesis could prevent cell proliferation. Therefore, restoration of the UMP level by the salvage pathway would overcome the ability of an inhibitor of the *de novo* pathway to prevent proliferation. To some extent, the degree of cytidine blockade may be dependent on the flux through the salvage pathway.

There are six enzymes responsible for the synthesis of UMP in the *de novo* pathway: CPS (glutamine-utilizing, EC 2.7.2.9), ATC (EC 2.1.3.2), and DHOase (EC 3.5.2.3) in the trifunctional CAD complex; DHODH in the mitochondria; and OPRT (EC 2.4.2.10) and OMPDC (EC4.1.1.23) in the bifunctional UMP synthase complex. We propose that one or more of them could be the target of leflunomide action. Each enzyme was tested against the active metabolite RS-61980, and only DHODH, the fourth enzyme in the pathway, was inhibited. This is the first report providing direct evidence for the mechanism of action of leflunomide. The identification of DHODH as a target of the action of leflunomide provides not only the foundation for understanding its inhibition of immune responses, but also guidelines for developing compounds with improved potency and selectivity.

MATERIALS AND METHODS

Reagents

RS-61980, RS-56338 (2-hydroxyethylidene-cyanoacetic acid-4-trifluoromethyloxy anilide), and Brequinar were synthesized at Syntex Discovery Research. All other chemicals were purchased from Sigma (St. Louis, MO). Radiochemicals were purchased from Moravek (Brea, CA).

Cell culture and measurement of proliferation

Human PBL were separated from heparinized blood of normal volunteers by centrifugation in Ficoll-Paque as described [12]. A3.01 cells (human T-lymphoblastoma) and PBL were cultured in RPMI 1640 with 5% fetal bovine serum as described [15]. For proliferation studies, A3.01 cells were seeded at a density of 1×10^5/mL. Test compounds and nucleosides were dissolved in DMSO and added at the time of seeding. The final DMSO concentration in the culture was 0.2%. To monitor proliferation, the number of cells was determined by direct counting with a hemocytometer. The cells were counted every 24 hr up to 120 hr, and the data at 72 hr were used to determine the effects of treatments.

Source of enzymes

A3.01 cells in log phase were harvested and washed in phosphate-buffered saline. Cytosolic extracts and mitochondrial lysates were prepared and used in the enzyme assays as described below.

CPS, ATC and DHOase. Fresh A3.01 cells were homogenized in 5 vol. of 2 mM potassium phosphate, pH 7, containing 40% DMSO, 10% glycerol, 0.5 mM EDTA and 1 mM dithiothreitol [16]. Membranes and particulate materials were removed by centrifugation at 15,000 *g* for 15 min. The resulting supernatant (cytosolic extract) was used as the source of the enzymes. The cytosolic extracts were prepared on the same days as the enzyme assays.

DHODH. Fresh A3.01 cells were resuspended in 9 vol. of 0.25 M sucrose and ruptured by a Dounce ho-

mogenizer. Intact mitochondria were prepared as described [17]. Mitochondria were solubilized in 0.1 M Tris–Cl, 10% glycerol and 0.03% Lubrol and used as the source of the enzyme [18].

OPRT and OMPDC. The cytosolic extracts of A3.01 cells were prepared as described above for CPS, ATC and DHOase except for one modification, which was omitting DMSO from the lysis buffer.

Cytidine deaminase (EC 3.5.4.5). Cytidine deaminase activity was measured from both intracellular and extracellular sources. For intracellular levels of enzyme, the cytosolic extracts of A3.01 cells and human PBL were prepared exactly as described above for OPRT and OMPDC. To measure extracellular cytidine deaminase, culture medium (72 hr after seeding) was used as a source of enzyme.

Determination of enzyme activity

CPS. The activity of CPS was determined by measuring the formation of [^{14}C]carbamyl aspartate in a coupled reaction. Reaction mixtures consisted of 50 mM potassium-HEPES, pH 7.4, 10% glycerol, 2 mM dithiothreitol, 10 mM magnesium chloride, 1 mM ATP, 1 mM glutamine, 5 mM sodium bicarbonate and 90 μM L-[U-^{14}C]aspartic acid (30 mCi/mmol). Cytosolic extracts (25 μg protein), which contained CPS, ATC and other enzymes, were added to start the reaction (final reaction volume of 50 μL). This reaction mixture allowed the formation of carbamyl phosphate catalyzed by CPS and the subsequent formation of [^{14}C]carbamyl aspartate catalyzed by ATC. Since exogenous carbamyl phosphate was not added to the reaction mixtures, [^{14}C]carbamyl aspartate could only be formed as a result of the CPS reaction. For a measurement of the background level of [^{14}C]carbamyl aspartate formation, substrates (magnesium ion, ATP, glutamine and sodium bicarbonate) required for CPS were omitted from the reaction mixture. This level was confirmed to be low (less than 2%).

After 30 min at 40°, a solution containing carriers was added, and the reaction was stopped by heating the samples at 100° for 3 min. The carriers were 1 μmol aspartic acid, 2.5 μmol carbamyl aspartate, and 0.04 μmol dihydroorotic acid. Radioactive compounds were separated by ion-pairing HPLC using a C18 Microsorb column (Rainin, Emeryville, CA). The materials of interest were eluted at a flow rate of 0.5 mL/min with 40 mM potassium phosphate, pH 6, 5 mM tetrabutylammonium phosphate (IPC-A reagent, Alltech Associates, Deerfield, IL). Under these conditions, the retention time values were 3, 5.2, and 7.4 min for aspartic acid, dihydroorotic acid, and carbamyl aspartate, respectively. The carriers marked the elution positions of these metabolites, and the elution profile was monitored by optical density at 230 nm. Eluents were collected, and the amounts of radioactivity co-migrating with each carrier were measured by liquid scintillation counting.

ATC. ATC activity was assayed by the formation of carbamyl aspartate using a colorimetric method. Reaction mixtures consisted of 100 mM Tris–Cl, pH 9, 2.5 mM L-aspartic acid and 0.5 mM carbamyl phosphate. Cytosolic extracts (25 μg protein) were added to start the reaction (final volume of 100 μL). After 20 min at 40°, the samples were stopped with 300 μL of 5% trichloroacetic acid. Cytosolic proteins were allowed to precipitate for 30 min at 0° and then were removed by centrifugation. The resulting supernatant, which contained the product of the reaction, was treated with an equal volume of a color reagent (antipyrine/sulfuric acid and diacetyl monoxime/acetic acid) for the detection of ureido compounds. The procedures of Prescott and Jones [19] were followed exactly. Briefly, the color development was carried out in the dark for 17 hr at room temperature and then in the light for 70 min at 45°. The absorbance at 466 nm was measured immediately. Under these conditions, carbamyl phosphate did not give a signal. For the measurement of the background level of ureido compounds in the assay, reaction samples were prepared where either carbamyl phosphate or aspartic acid was omitted. The background of this assay procedure was insignificantly low (less than 1%).

DHOase. DHOase was assayed by the formation of carbamyl aspartate in the reverse reaction. Reaction mixtures consisted of 100 mM Tris–Cl, pH 8.5, and 20 μM dihydroorotic acid. The assay conditions and procedures of detecting carbamyl aspartate were exactly as described above for ATC.

DHODH. The activity of DHODH was measured by the formation of [^{14}C]orotic acid from [^{14}C]dihydroorotic acid. The reaction mixtures consisted of 75 mM Tris–Cl, pH 7.8, 5 mM potassium cyanide, 0.6 mM coenzyme Q (Q30), and 10 μM L-[2-^{14}C]dihydroorotic acid (50 mCi/mmol), as described [18]. The reaction was initiated by the addition of mitochondrial lysate (1.6 μg protein). After 10 min at 40°, a carrier solution was added, and the reaction was stopped by heating at 100° for 3 min. The carrier solution contained 50 nmol dihydroorotic acid and 12.5 nmol orotic acid. The radioactive product and substrate were separated by ion-pairing HPLC and quantitated by liquid scintillation counting as described above for CPS. A significant level of [^{14}C]orotic acid formation was detected in the absence of coenzyme Q (see Results and Fig. 6).

OPRT and OMPDC. The two enzymes were assayed together in the same reaction. The assay mixture consisted of 40 mM Tris–Cl, pH 8, 5 mM magnesium chloride, 50 μM PRPP and 10 μM [5-^{3}H]orotic acid (0.1 Ci/mmol). The reaction was initiated with cytosolic extracts (100 μg protein) in a final volume of 100 μL. After 15 min at 40°, a carrier solution was added, and the reaction was stopped by heating at 100° for 3 min. The carrier solution consisted of 10 nmol each of orotic acid, OMP and UMP. The radioactive compounds were separated by HPLC using a Partisil 10 SAX column (Whatman) with a gradient of 20 mM (buffer A) to 50 mM (buffer B) potassium phosphate, pH 5.9, as follows: 0 to 15 min, 0% B; 15 to 30 min, linear gradient to 100% B. The flow rate was 1 mL/min. Under these conditions, the retention time values were 4.8, 10.3, and 50.7 min for orotic acid, UMP and OMP, respectively. The elution profiles of the carriers were monitored by optical density at 260 nm. The radioactive eluents were collected and then quantitated by scintillation counting.

Cytidine deaminase. Cytidine deaminase associated with A3.01 cells or human PBL was measured by the formation of [^{3}H]uridine from [^{3}H]cytidine. The reaction mixture consisted of 20 mM Tris–Cl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol and 0.2 mM [5-^{3}H]cytidine (0.1 Ci/mmol). Cytosolic extract (13–37 μg protein) or culture medium (26–31 μg protein) was added to initiate the reaction, which proceeded at 40° for 30 min. A carrier solution that contained 5 nmol each of cytidine and uridine was added to the mixture, and the reaction was

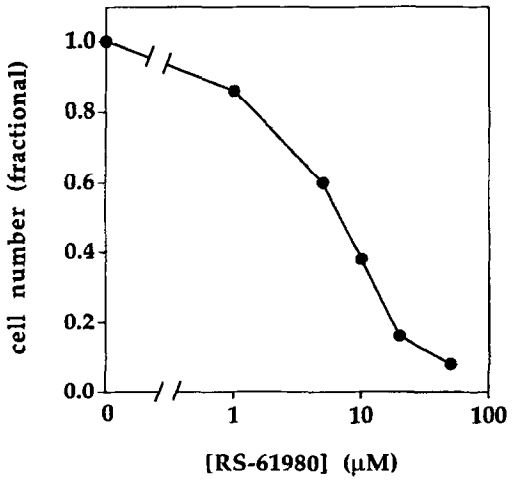


Fig. 2. Growth inhibition of A.301 cells by RS-61980. A3.01 cells were seeded at 1×10^5/mL, and proliferation was monitored by the number of cells. RS-61980 was added at the time of seeding. The cell number, after 72 hr, was determined by direct counting. The data were fitted to the computer program Systat for IC_{50} determination. Cell viability was monitored by trypan blue exclusion. Cell death was typically less than 3% at the highest inhibitor concentration.

stopped by heating at 100° for 3 min. Radioactive cytidine and uridine were separated by HPLC using a C18 Microsorb column eluted with 5 mM potassium phosphate, pH 5.9, at a flow rate of 1 mL/min. The retention time values for cytidine and uridine were 6 and 8.6 min, respectively. The radioactive product and substrate were quantitated as described above.

Determination of inhibition constants

Inhibition and kinetic constants were determined by fitting the initial velocity data to appropriate equations using nonlinear regression analysis by the computer program Systat. Determinations are reported as "value ± asymptotic standard error."

RESULTS

Effect of RS-61980 on cell proliferation

RS-61980 is the primary metabolite of leflunomide *in vivo*, and it was used in place of leflunomide in the experiments presented in this manuscript. RS-61980 inhibited the proliferation of a human T-lymphoblastoma cell line (A3.01) with an IC_{50} of 6.5 ± 0.6 μM (Fig. 2). At a 25 μM concentration of this compound, the inhibition was overcome completely by uridine (Fig. 3a). Under these conditions, cytidine was slightly effective (Fig. 3b), whereas guanosine and adenosine were not effective in overcoming the inhibitory effect (data not shown). At 100 μM RS-61980, the inhibition of proliferation could still be overcome by uridine, although not completely (data not shown). These results are consistent with the hypothesis that RS-61980 affects pyrimidine nucleotide metabolism. We reasoned that since uridine can be readily converted to UMP by the salvage enzyme uridine/cytidine kinase, it is likely that RS-61980 interferes with the *de novo* pathway of UMP synthesis. Therefore, if UMP is replenished by uridine through the salvage pathway, the inhibitory effect of RS-61980 is abrogated.

This result with the A3.01 cells, where cytidine was unable to overcome growth inhibition as efficiently as uridine, is in contrast to results with human PBL, where the growth inhibition caused by RS-61980 was overcome completely by either uridine or cytidine. Since cytidine can be readily deamidated to uridine by cytidine deaminase, it is likely that the blocking effect of cytidine in PBL is through uridine. To test the possibility that cytidine deamination occurred more efficiently in PBL than in the A3.01 cells, cytidine deaminase activity associated with both types of cells was determined. We found that the intracellular enzyme activity in PBL was nearly 500 times higher than that of A3.01 cells. In PBL, the specific activity was 5 nmol uridine produced/min/mg of cytosolic protein; in A3.01 cells, it was less than 9 pmol uridine produced/min/mg cytosolic protein. We also detected cytidine deaminase activity in the culture medium of the PBL at a specific activity of 0.16 nmol uridine produced/min/mg of serum protein. No cytidine deaminase activity was detected in the medium of the

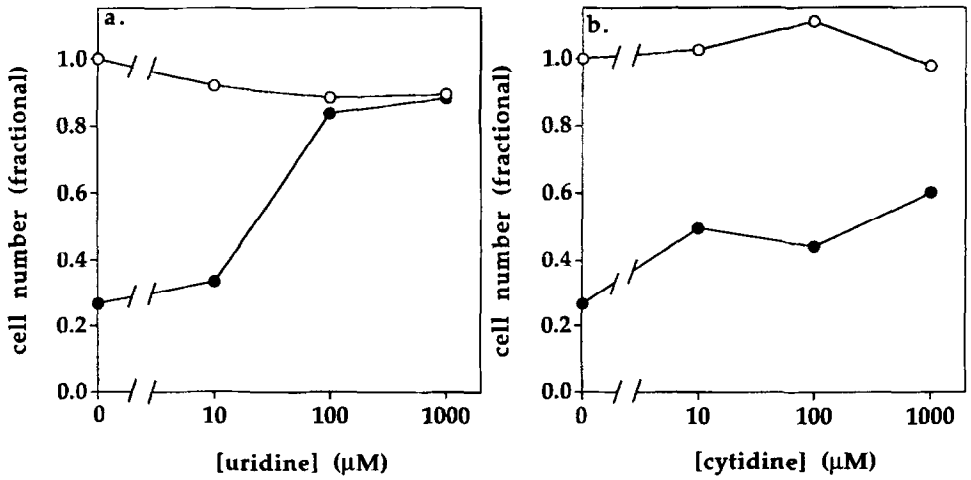


Fig. 3. Blocking of RS-61980 action by nucleosides. A3.01 cell proliferation was monitored as described in the legend of Fig. 2. Nucleosides [uridine (a) or cytidine (b)] and RS-61980 were added at the time of seeding. Cells were treated with either 25 μM (●) or no (○) RS-61980. The ordinate of each panel represents the relative amount of surviving cells.

Table 1. Effect of RS-61980 on the *de novo* enzymes of UMP synthesis

Enzyme	RS-61980 (μM)	% Inhibition	Reference inhibitor	K_i observed	K_i expected
CPS	500	5	Acivicin	139 ± 10 μM	105 μM
ATC	400	0	Phosphonoacetic	6.2 ± 4.6 mM	0.5 mM
DHOase	200	0	5-Amino orotic acid	47.3 ± 0.8 μM	28 μM
DHODH	100	96	Brequinar	0.46 ± 0.3 nM	5–8 nM
OPRT	200	0	6-aza-UMP	7.3 ± 0.7 μM	20 μM
OMPDC	200	7	6-aza-UMP	140 ± 120 nM	100 nM

The percent inhibition values represent the averages of 2–4 experiments. The observed K_i values (mean ± asymptotic standard error, N ≥ 3) towards each reference inhibitor were measured as described in Materials and Methods. The expected K_i values were from previous publications: CPS [20], ATC [20], DHOase [20], DHODH [18], OPRT [21] and OMPDC [20].

A3.01 cells, which was identical to the PBL medium at the time of seeding. These data support the interpretation that the ability of cytidine to overcome the action of RS-61980 is related to the level of cytidine deaminase.

Effect of RS-61980 on enzymes in the de novo synthesis of UMP

To test the hypothesis that leflunomide exerts its immunosuppressive activity by inhibiting the *de novo* synthesis of UMP, we examined the activity of RS-61980 against each of the six enzymes in this pathway. The activity of each enzyme was assayed separately, and the effect of RS-61980 was determined (Table 1). The only enzyme that was inhibited by the compound was DHODH, the fourth enzyme in the pathway. DHODH is located in the mitochondria and catalyzes the oxidation of dihydroorotic acid to orotic acid using coenzyme Q as an electron acceptor. The other five enzymes, CPS, ATC, DHOase, OPRT and OMPDC, were not affected by RS-61980 up to 200–500 μM. To ascertain that the activity assays were sensitive enough to detect even weak inhibition, the activity of RS-61980 was compared with that of known inhibitors of each enzyme. Under our assay conditions, the K_i values of these reference inhibitors were generally similar to previously reported values. These results verify that the only step in the *de novo* pathway inhibited by RS-61980 is the oxidation of dihydroorotic acid to orotic acid.

Inhibition of DHODH by RS-61980

RS-61980 inhibited DHODH with a K_i of 2.7 ± 0.7 μM (Fig. 4). Leflunomide inhibited DHODH with an observed K_i of 4.6 ± 2.4 μM; RS-56338, an analog of RS-61980, was also active against DHODH with a K_i of 1.8 ± 0.5 μM (see Fig. 1 for structure; data not shown). RS-56338 had a potency similar to that of RS-61980 in inhibiting cell proliferation (IC_{50} of 4.6 ± 0.2 μM).

RS-61980 exhibited mixed-type inhibition kinetics towards both substrates of DHODH (Fig. 5). The K_{ii} and K_{is} values were 1.16 ± 0.35 and 3.28 ± 2.45 μM respectively, for dihydroorotic acid, and 2.62 ± 1.36 and 0.69 ± 0.39 μM, respectively, for coenzyme Q.

During the course of measuring the concentration dependence of RS-61980 and RS-56338 inhibition, we reproducibly observed that a certain portion of the orotic acid forming activity was resistant to the inhibition by either RS-61980 or RS-56338. This residual activity was also resistant towards Brequinar up to 100 μM, which is 100,000 times higher than the K_i. Interestingly, this activity could produce orotic acid from dihydroorotic acid, independent of coenzyme Q (Fig. 6).

DISCUSSION

Leflunomide is rapidly metabolized *in vivo*, where the primary metabolite RS-61980 is detected in plasma within minutes after administration of the drug [13]. To investigate the mechanism of action of leflunomide, each enzymatic step of the *de novo* synthesis of UMP was examined. Using RS-61980, we demonstrated that only the fourth enzyme in the pathway, DHODH, was inhibited. The kinetic mechanism of inhibition was determined to be a mixed type towards both substrates, dihydroorotic acid and coenzyme Q. Since there is no striking structural resemblance between the compound and either substrate, it is likely that RS-61980 interacts with the enzyme at a site distinct from the substrate binding sites. DHODH is the target for Brequinar, which also exhibits mixed-type inhibition towards both substrates

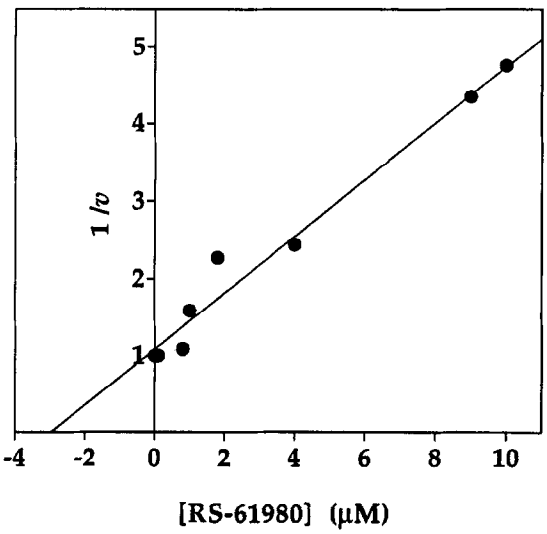


Fig. 4. Inhibition of DHODH by RS-61980. Mitochondria were solubilized and enzyme was assayed as described in Materials and Methods. The initial velocity data were normalized to maximum velocity and presented in a Dixon plot of reciprocal fractional velocity versus inhibitor concentration. The computer program Systat was used for the determination of K_i. Coenzyme Q-independent activity was subtracted (see the legend of Fig. 6).

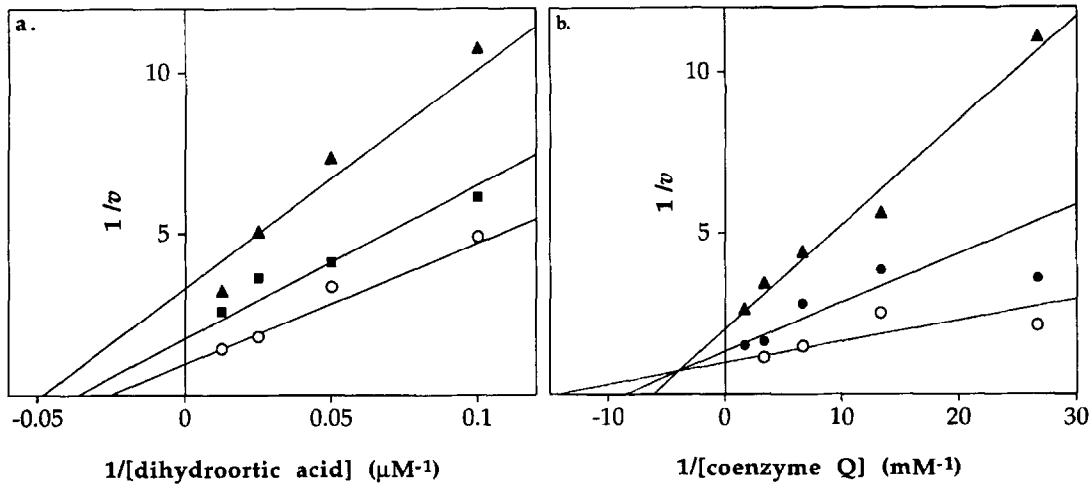


Fig. 5. Kinetics of DHODH inhibition by RS-61980. Inhibition of DHODH by RS-61980 is presented in double-reciprocal plots. Enzyme activity was measured as described in the legend of Fig. 4. RS-61980 was present at 0 μM (○), 0.9 μM (■) and 2.7 μM (▲). (a) Dihydroortic acid was the varying substrate and coenzyme Q was present at 0.6 mM. (b) Coenzyme Q was the varying substrate and dihydroorotic acid was present at 100 μM. The initial velocity was normalized to maximum velocity. The fractional velocity was fitted to the equation of Cleland for noncompetitive inhibition. The lines represent the theoretical data based on the fit. These data represent one of three experiments, all of which showed similar results.

[22]. Kinetic analysis of Brequinar inhibition of DHODH suggests that the compound binds to the enzyme at a unique site that is distinct from the substrate binding sites [22]. Currently, it is unknown whether Brequinar and RS-61980 bind to the same site.

The effectiveness of leflunomide in DHODH inhibition was examined and was found to be similar to that of RS-61980. Based on the structural similarities, it is perhaps not surprising that the two are similar in potencies. However, the determination of K_i is somewhat complicated by the fact that leflunomide can be metabolically converted to RS-61980. Since mitochondrial lysates were used as the source of enzyme, it is possible that some conversion took place during the enzyme assay, and the observed K_i value represents the potency of a mixture of both compounds. To address this uncertainty, the time dependence of inhibition was examined. We reasoned that if leflunomide was metabolized during the assay, and if it was less potent than RS-61980, the degree of inhibition would increase with time of reaction as RS-61980 accumulated. Conversely, if leflunomide was more potent than RS-61980, the inhibition would decrease with time as it was metabolized. We found that inhibition of DHODH by leflunomide increased slightly with time (data not shown). When the reaction time and temperature were lowered to 15 sec and 25°, the K_i was approximately 10 μM (twice the value for a 10-min reaction at 40°). This result suggests that while leflunomide may be metabolized during the enzyme assay, it is active towards DHODH. However, an accurate determination of the K_i of leflunomide awaits repeating the experiment with purified enzyme.

DHODH is an enzyme capable of utilizing either molecular oxygen (oxidase) or coenzyme Q (dehydrogenase) as electron acceptor [23]. Lakaschus and Löffler [24] demonstrated that the two activities are differentially inhibited by Brequinar, to which the dehydrogenase is 500,000 times more sensitive than the oxidase. In our experiments, a small but significant amount of orotic acid formation in the mitochondria occurred in the absence of added coenzyme Q (Fig. 6). Since DHODH is the only enzyme known to oxidize dihydroorotic acid to orotic acid, we believe that this coenzyme Q-independent activity represents an "oxidase" activity of DHODH. Interestingly, leflunomide inhibition of DHODH was also much weaker towards the coenzyme Q-independent activity.

Uridine was effective in completely preventing the antiproliferative effect of RS-61980 when the concentration of the drug was at 25 μM (Fig. 3a). The completeness of the block is an indication of the selectivity of leflunomide inhibition on *de novo* UMP synthesis. However, at 100 μM RS-61980 (15 times the IC_{50}), the antiproliferative effect was partially overcome by uridine (data not shown). This result indicates that while the main action of RS-61980 may be the inhibition of *de novo* UMP synthesis, there may be side effects that are less profound and unrelated to pyrimidine nucleotide

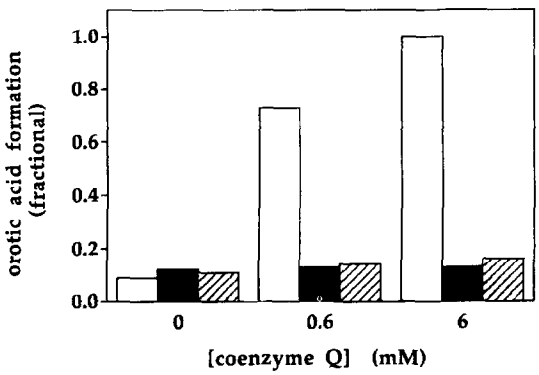


Fig. 6. Coenzyme Q-independent activity of DHODH. Orotic acid formation was determined as described in Materials and Methods. Key: 100 μM RS-61980 (■), 100 μM Brequinar (▨); and no inhibitor (□). The fractional activity of 1.0 represents the data from 6 mM Q. These data represent one of three experiments, all of which showed similar results.

metabolism. These "side effects" occur at higher concentrations of the drug. As a comparison, Brequinar at 500 nM (12.5 times the IC_{50}, which is 40 nM) was completely overcome by uridine (data not shown). Using the uridine effect as a criterion for selectivity, leflunomide appears to be less selective than Brequinar. RS-61980 is less potent than Brequinar in both cell growth and DHODH inhibition by three orders of magnitude.

In summary, this study suggests a molecular target for leflunomide, a novel and clinically effective immunosuppressive agent. This should enable further efforts to develop compounds with improved potency and selectivity.

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