Purification of human dihydro-orotate dehydrogenase and its inhibition by A77 1726, the active metabolite of leflunomide

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Leflunomide is currently in phase-III clinical trials for the treatment of rheumatoid arthritis. In this study, we have focused our efforts on the study of the mechanism of action of the active metabolite of leflunomide, A77 1726, in cells and tissue of human origin. The human high-affinity binding protein for radiolabelled A77 1726 was purified from solubilized U937 membranes by following the binding activity through the purification process and was characterized as the mitochondrial enzyme dihydro-orotate dehydrogenase (DHO-DH). The human and murine enzyme displayed identical pI and molecular mass values on SDS/PAGE (43 kDa), which contrasts notably with previous reports suggesting a molecular mass of 50 kDa for the human enzyme. DHO-DH activity was inhibited by A77 1726 and its analogue HR325 with similar potency in U937 and human spleen membrane preparations. HR325 was found to be anti-proliferative for phytohaemagglutinin-stimulated human peripheral blood mononuclear cells, at the same concentrations that caused accumulation of DHO and depletion of uridine. Supplementation of the cultures with exogenous uridine led to partial abrogation of the anti-proliferative effect. This is in line with our recent demonstration that the anti-proliferative effect *in vitro* of A77 1726 on lipopolysaccharide-stimulated mouse spleen cells was mediated by DHO-DH inhibition [Williamson, Yea, Robson, Curnock, Gadher, Hambleton, Woodward, Bruneau, Hambleton, Moss et al., (1995) J. Biol. Chem. **270**, 22467–22472]. Thus, DHO-DH inhibition by A77 1726 and its analogues is responsible for the anti-proliferative effects *in vitro* of the compounds on human cells and is likely to be responsible for some of its effects *in vivo*.

INTRODUCTION

Leflunomide [*N*-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide] is an immunomodulatory compound that shows activity in a range of immunologically driven models of inflammation and transplantation [1]. It has shown clinical efficacy against arthritis [2] and is currently undergoing phase-III clinical trials for this indication. Leflunomide is transformed *in vivo* very rapidly to its active metabolite A77 1726, which is active both *in vitro* and *in vivo*. We have shown previously that both A77 1726 and HR325, a structural analogue of A77 1726, bind with high affinity to the mouse mitochondrial enzyme dihydro-orotate dehydrogenase (DHO-DH, E.C 1.3.3.1) and inhibit its activity, which is the catalysis of the fourth step of pyrimidine biosynthesis *de novo*. Inhibition of enzyme activity correlates with anti-proliferative effects that can be overcome both *in vivo* and *in vitro* by uridine supplementation [3].

The clinical use of leflunomide motivated our attempt to identify its human target for drug action. The promyelomonocytic U937 cell line was chosen as a source of purification material for its binding characteristics and large-scale culture properties. High-affinity binding of radiolabelled A77 1726 was used to follow the purification procedure. A 43 kDa protein was purified to near homogeneity and identified as the human DHO-DH. Additional studies showed similar characteristics of DHO-DH from human spleen and from U937. These results contrast with a published report which suggested that the human enzyme has an atypical subunit molecular mass of 50 kDa [4].

The presented data show that A77 1726 and its analogue inhibit DHO-DH in human cells, resulting in accumulation of substrate, depletion of uridine levels and inhibition of proliferation. These results are in support of the view that inhibition of DHO-DH is a mechanism of action that may be relevant for the therapeutic effects of leflunomide.

MATERIALS AND METHODS

Materials

2,6-Phenyl [^{3}H]A77 1726 (34.3 and 51.4 Ci/mmol) and its iodoazido analogue [^{125}I]RU35072 (Figure 1; 2000 Ci/mmol; [3]) were radiolabelled in the Laboratoire de Marquages Isotopiques, Roussel-UCLAF, Romainville, France. Unlabelled test compounds including A77 1726 and HR325 (Figure 1) were synthesized in the Chemistry Department, Hoechst Marion Roussel Ltd., Swindon, U.K. Trifluoroacetic acid was from Applied Biosystems, endoproteinase Asp-N was from Boehringer. Octyl glucoside, coenzyme Q10, dihydro-orotic acid and dichlorophenol-indophenol (DCIP) were obtained from Sigma.

Cell culture

Mycoplasma-free U937 cells were maintained in semi-continuous culture in RPMI 1640 supplemented with 5% fetal calf serum/4 mM glutamine/1 mM sodium pyruvate/0.2% sodium bicarbonate/0.1% glucose/100 units/ml penicillin/100 μg/ml streptomycin. Cells were harvested during log-phase growth, washed with Dulbecco's PBS and frozen at −80 °C.

Abbreviations used: DHO-DH, dihydro-orotate dehydrogenase; DCIP, dichlorophenol-indophenol; RU35072, ^{125}I-labelled iodoazido-analogue of A77 1726; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; PBMC, peripheral-blood mononuclear cell.

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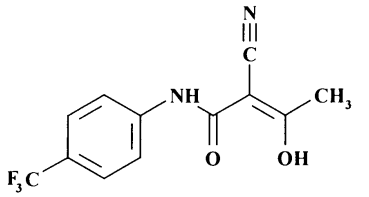
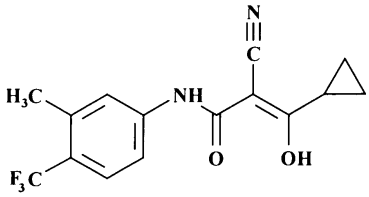
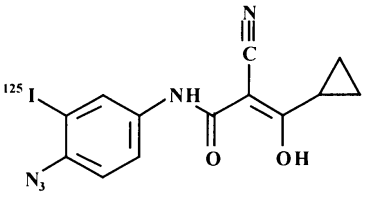


A77 1726



HR325



RU 35072

Figure 1 Chemical structures of A77 1726, HR325 and RU35072

Radioligand binding assay

[^{3}H]A77 1726 binding of U937 and human spleen membranes was assessed as described elsewhere [3]. Binding data were analysed with EBDA and LIGAND programs [5].

Preparation and solubilization of U937 and human spleen membranes

Human spleen membranes were prepared as described [3]. Frozen U937 cells were homogenized in 20 mM Tris/HCl, pH 7.2, containing 1 mM EDTA (1×10^8 cells/0.37 ml; all buffers were precooled containing protease inhibitors), and cell debris was removed by centrifugation (2000 ***g***, 10 min). The mitochondrial/microsomal membranes were collected from the supernatant by centrifugation at 160000 ***g*** for 1 h at 4 °C and washed with buffer containing 125 mM sucrose and 150 mM NaCl. After centrifugation, the pellet was either used in binding studies or solubilized in 20 mM Tris/HCl containing 150 mM NaCl, 1 mM EDTA and 1 % octyl glucoside on ice for 1 h. Particulate matter was removed by centrifugation at 100000 ***g*** for 1 h at 4 °C.

Purification of the U937 A77 1726-binding protein

Crude solubilized preparation (1–2 g protein) was diluted 4-fold into 20 mM Mops, pH 7.2, containing 1 % octyl glucoside and 1 mM EDTA and loaded on to a previously equilibrated column of S-Sepharose HP (10 mm × 5 cm) at a flow rate of 20 ml/min. The column was washed with the same buffer until A_{280} returned to baseline and then eluted with a linear gradient from 0 to 450 mM NaCl, incorporating a plateau at 50 mM NaCl. The peak A77 1726-binding fractions were collected (200–250 mM NaCl) and applied at 1 ml/min to an HCA hydroxyapatite column (7.6 mm × 10 cm; Mitsui Toatsu Chemicals Inc.) previously equilibrated with 5 mM KH_2PO_4 (pH 7.0)/1 % octyl glucoside. The column was washed with the same buffer and eluted with a linear gradient from 5 to 500 mM KH_2PO_4 and peak fractions were either stored at −80 °C for use in enzyme assays or desalted on PD-10 columns (Pharmacia) into chromatofocusing elution buffer [a 1:45 dilution of Pharmalyte (pH 8–10.5)/1 % octyl glucoside (pH 7.0)] for further purification. A 9 mm × 30 cm column of PBE 118 polybuffer exchanger was equilibrated with 600 ml of 25 mM triethylamine/HCl, pH 11, containing 1 % octyl glucoside. Following application of 5 ml of elution buffer, the desalted sample was applied at 0.5 ml/min and eluted with 165 ml of elution buffer. Remaining protein was eluted with a 1 M NaCl wash. Eluate fractions were stored at −80 °C.

Gel filtration was performed using a Superose 12 HR 10/30 column on an FPLC system (Pharmacia). Samples (0.2 ml) were loaded and eluted at 0.5 ml/min in a buffer containing 25 mM Tris/HCl, 0.5 % nonyl glucoside, 150 mM NaCl, pH 7.4. Column retention was calibrated using proteins ranging in molecular mass from 6.5 to 200 kDa.

Protein concentrations were determined using the bicinchoninic acid method (Pierce) using a purified immunoglobulin as standard.

Proteolytic digestion and internal sequence analysis

The purified A77 1726 binding site was concentrated by chloroform/methanol extraction [6], resuspended in 200 μl electrophoresis sample buffer (62.5 mM Tris/HCl/3 % SDS/10 % β-mercaptoethanol/10 % glycerol, pH 6.8) and heated for 5 min at 100 °C. After electrophoresis in a 10 % polyacrylamide gel [7], the 43 kDa protein band was localized by staining with 0.1 % Coomassie Brilliant Blue in 20 % methanol/0.5 % acetic acid, partially destained with 30 % methanol, and finally excised. The gel slice was then fully destained in 4 × 1 ml of 100 mM ammonium bicarbonate (pH 8.0)/50 % acetonitrile at 56 °C (each for 20 min), dried in a Speed-Vac concentrator and rehydrated with 50 μl of 100 mM ammonium bicarbonate, pH 8.0, containing 0.02 % Tween 20, and further ammonium bicarbonate to full rehydration without liquid excess. Asp-N endoproteinase digestion (0.16 μg in 50 μl of 100 mM ammonium bicarbonate, pH 8.0) was performed for 18 h at 37 °C with occasional vortexing.

Peptides were extracted from the gel with four 500 μl aliquots of 60 % acetonitrile containing 0.1 % trifluoroacetic acid at 56 °C (20 min per extraction). Extracts were pooled, Speed-Vac-concentrated to 200 μl, and subjected to reversed-phase chromatography on an RP300 1 × 100 mm column. Peak fractions were concentrated to 50 μl, applied to PVDF membranes (Prospin, Applied Biosystems) and sequenced directly in a model 473A microsequencer cartridge (Applied Biosystems).

Photoaffinity labelling and SDS/PAGE

Photoaffinity labelling was performed as described in [3] using 1 nM [^{125}I]RU35072. High-affinity binding was defined by competition with 1 μM HR325. Following labelling, proteins were precipitated with 20 % trichloroacetic acid, washed with acetone

and, following solubilization in electrophoresis sample buffer, were subjected to SDS/PAGE in 12.5 % acrylamide as described above. Proteins were visualized by Coomassie Brilliant Blue staining, dried and autoradiographed using X-ray film (Hyperfilm MP, Amersham) and two intensifying screens (Quanta III-T, Dupont) at −80 °C for 5 days.

Assay of DHO-DH activity in membrane fractions

DHO-DH activity in membrane preparations was assayed by the DHO-driven reduction of DCIP [9]. Membranes were pre-incubated in 50 mM Tris/HCl containing 0.1 % Triton X-100, 1 mM KCN, 100 μM coenzyme Q10 and 200μM DCIP for 30 min (U937; 0.43 mg of protein/ml) or 90 min (spleen; 2.5 mg of protein/ml). The reaction was initiated by the addition of 500 μM DHO and the reduction of DCIP monitored by measurement of the absorbance at 650 nm in a 96-well kinetic plate reader at 37 °C for 10–15 min. The linear rate of reduction of DCIP was determined and the rate of orotate production calculated using an extinction coefficient of $\epsilon_{650} = 12.26\ mM^{-1}$ (path length = 0.57 cm).

Measurement of intracellular DHO and uridine concentrations

Cell lysates were analysed by HPLC. DHO was analysed using an AS 11SC ion-exchange column (25 cm × 4.6 mm). Samples were applied in water and eluted with a gradient of NaOH (0–60 mM). Eluted DHO was detected by conductance. Uridine was separated on a Hypersil ODS column (25 cm × 4 mm). Samples were applied in 10 mM KH_2PO_4, pH 5.5, and eluted using a gradient of methanol (0–20 %). Uridine was detected by absorbance at 254 nm.

Elution characteristics of DHO and uridine were determined by injection of known concentrations of the pure compounds. Quantification of DHO and uridine in unknown samples was done by measuring the area of the eluted peaks at relevant time points and comparing with standard curves.

RESULTS

U937 binding-site characterization

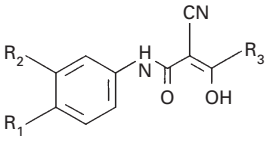
Competition binding experiments using a fixed concentration of [^{3}H]A77 1726 (25 nM) and increasing concentrations of unlabelled A77 1726 (0.1 nM–1 mM) or HR325 (2 nM–458 μM) were performed on different U937 membrane preparations. In all cases unlabelled ligand displaced radioligand in a dose-dependent manner, yielding shallow displacement curves characteristic of binding to more than one site. The binding isotherms from homologous competition studies were best described by two-site fits (Table 1) predicting sites of high (68 nM) and low (135 μM) affinities. HR325 displaced [^{3}H]A77 1726 with a similar affinity to A77 1726 at high (51 nM) and low (209 μM) affinities.

Purification and characterization of the high-affinity U937 A77 1726-binding protein

High-affinity binding of [^{3}H]A77 1726 was used to follow the purification process. It was defined as an approximately 50 % displacement by 2 μM unlabelled A77 1726 and 95 % of the binding in S-Sepharose HP and hydroxyapatite fractions, respectively. Sequential chromatography (S-Sepharose HP, hydroxyapatite and chromatofocusing columns) of solubilized membranes from 2×10^{11} U937 cells yielded approximately 105 pmol of protein with more than 5000-fold purification. The A77 1726-binding peak eluted at a pH of 9.8 from the chromatofocusing column and contained a single major band at a molecular mass of 43 kDa on SDS/PAGE (Figure 2). Gel filtration of hydroxyapatite-purified fractions yielded a native molecular mass for the DHO-DH activity of approximately 130 kDa. A band at 43 kDa was enriched from each stage of purification, as shown by photoaffinity labelling experiments, which were completely inhibited by 1 μM HR325 (Figure 2).

The chromatofocusing eluate was subjected to SDS/PAGE and, following excision, the 43 kDa protein was digested in-gel with endoproteinase Asp-N. The resultant peptides were separated by reversed-phase chromatography on a microbore C_8 column. Of the 20 peptides collected, two yielded unambiguous

Table 1 Inhibition of U937 and human spleen DHO-DH



			IC_{50} value*	
R_1	R_2	R_3	Human spleen	U937
CF_3	CH_3	Cyclopropyl	539 nM	813 nM
CF_3	H	CH_3	657 nM	676 nM
H	Cl	Thiophenyl	>100 μM	>100 μM
Brequinar sodium			3.6 nM	3.4 nM

* Calculated with respect to controls containing DMSO.

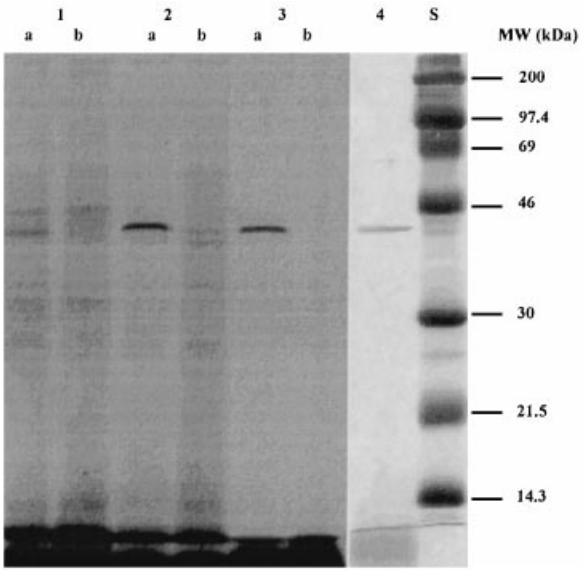


Figure 2 SDS/PAGE of fractionated U937 membranes

Purification samples were separated by SDS/PAGE as described in the Materials and methods section. Autoradiographs of photoaffinity-labelled S-Sepharose HP pool, hydroxyapatite pool and chromatofocusing eluate are shown in lanes 1, 2, and 3, respectively. Coomassie Brilliant Blue-stained chromatofocusing eluate is shown in lane 4, and molecular-mass standards (MW) are shown under S. For photoaffinity labelling, samples were incubated in the dark with the [^{125}I]iodoazido ligand prior to photolysis (lanes 1a, 2a and 3a). Parallel incubations, in which the photoaffinity ligand was prevented from binding to the high-affinity site by 1 μM HR325 (lanes 1b, 2b and 3b) were used to assess the specificity of the binding.

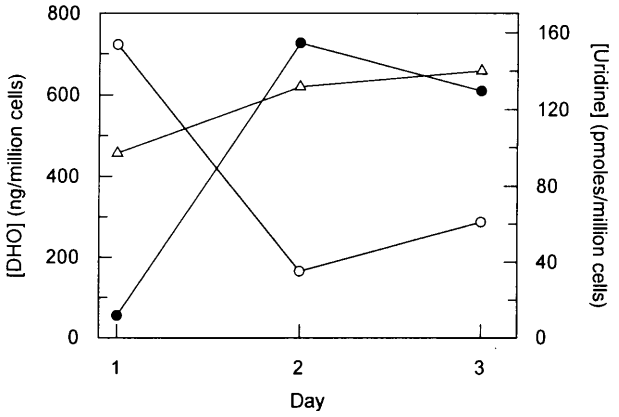


Figure 3 Effect of HR325 on intracellular levels of uridine and DHO

Human PBMCs were stimulated with 1 μg/ml PHA and treated with DMSO (△) or HR325 (●,○). On days 1–3 samples were removed and assayed for the presence of DHO (filled symbols) and uridine (open symbols). DMSO-treated cultures contained undetectable levels of DHO at all time points.

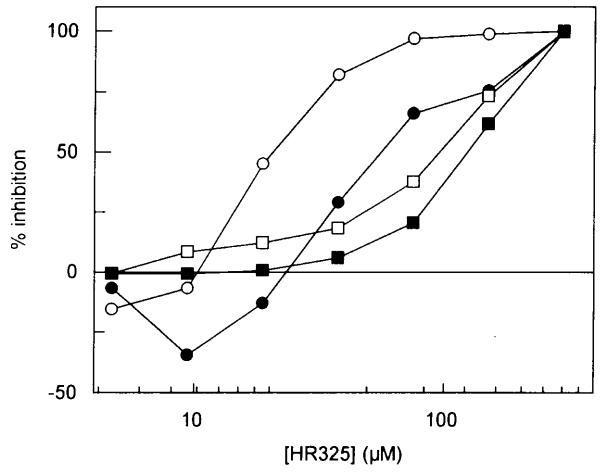


Figure 4 Effect of uridine supplementation on HR325 inhibition of proliferation

Proliferation of human PBMCs was stimulated by treatment with PHA (1 μg/ml) and interleukin-2 (100 units/ml). Proliferation was assessed by [^{3}H]thymidine incorporation on day 4. Percentage inhibition in the presence of varying concentrations of HR325 is shown with respect to control cultures containing the relevant concentration of uridine. ○, Without exogenous uridine; ●, 0.01 mM uridine; □, 0.1 mM uridine; ■, 1 mM uridine.

amino acid sequences (7 and 9 amino acids), both of which displayed complete identity with sequences from human DHO-DH (GLPLGVNL and DYAEGVR found at positions 176–183 and 195–201 respectively). Furthermore, DHO-DH activity co-purified with high-affinity [^{3}H]-A77 1726 binding through all stages of purification (results not shown).

Inhibition of U937 membrane and human spleen membrane DHO-DH by A77 1726 and selected analogues

A number of analogues of A77 1726 were tested for inhibition of DHO-DH activity on U937 and human spleen membranes using the DCIP colorimetric method. Human spleen samples (90 min) required longer preincubation than U937 membranes (30 min) to allow the endogenous reduction of DCIP [9] to subside. This difference was due, at least in part, to the higher protein concentration required of spleen membranes as a result of the lower specific activity of DHO-DH. In neither membrane preparation was the endogenous DCIP reduction sensitive to any of the compounds used (results not shown) and was effectively removed from the crude solubilized membranes by the S-Sepharose HP purification step.

All the compounds tested displayed similar IC_{50} values against U937 DHO-DH when compared with human spleen-derived DHO-DH activity (Table 1). Brequinar, a known potent inhibitor of DHO-DH that has undergone clinical evaluation for the treatment of cancer and organ-transplant rejection, was more active than A77 1726 and HR325 [10].

Purification and characterization of partially purified human spleen DHO-DH activity

Human spleen DHO-DH (81.2 g of tissue) was solubilized in nonyl glucoside, yielding 410 ml of supernatant with a specific activity of 2.1 units/ml (1 unit is defined as the amount of enzyme required to convert 1 nmol of DHO to 1 nmol of orotate under the conditions described). It was partially purified (54-fold over membranes) by cation-exchange chromatography on S-Sepharose HP as described previously for the mouse spleen activity [3] and eluted with a similar retention. The pooled peak fraction from the cation exchange contained 0.19 mg of protein/ml at a specific activity of 16.3 units/mg. Application of this sample to the gel-filtration column resulted in retention identical to that of U937 DHO-DH and predicted a molecular mass of 130 kDa.

Effect of HR325 on intracellular DHO and uridine concentrations

Human peripheral blood mononuclear cells (PBMCs) were stimulated with phytohaemagglutinin (PHA) in the presence of DMSO (vehicle), or 50 μM HR325 for 1, 2 or 3 days, whereafter DHO and uridine concentrations were measured (Figure 3). DHO could not be detected in DMSO-treated cultures at any time point. In the presence of HR325, DHO was detected on day 1 and increased rapidly to a maximum on day 2 (727 ng/1×10^6 cells). Uridine levels mirrored DHO levels and were inhibited by HR325 on days 2 and 3. A77 1726 caused similar effects to HR325 on both parameters (results not shown).

Effect of uridine supplementation on inhibition of proliferation

Human PBMCs were stimulated to proliferate by simultaneous treatment with 1 μg/ml PHA and 100 units/ml interleukin-2 and proliferation was measured by incorporation of [^{3}H]thymidine 4 days after stimulation. Figure 4 shows the dose-dependent inhibition of proliferation by HR325 in the absence and presence of uridine (0.01–1 mM). This experiment is 1 of 10 experiments performed on cells from different donors. The mean IC_{50} values for inhibition of proliferation were as follows: in the absence of added uridine, 32.5 ± 13.7 μM; in the presence of 0.01, 0.1 and 1 mM uridine, 115.1 ± 52.8 ($n = 10$), 111.9 ± 48.5 ($n = 10$) and 139 ± 29 μM ($n = 2$) respectively.

DISCUSSION

Leflunomide is currently in phase III-clinical studies. Therefore, experiments were carried out to elucidate the mechanism of action of the active leflunomide metabolite in humans. Similarly to mouse tissue, it was shown that A77 1726 action on human tissue was mediated through DHO-DH inhibition and that this enzyme was the only protein which bound A77 1726

with a high affinity. The radiolabelled A77 1726 binding was used to test a series of human cell lines for binding and to monitor the protein purification. Several cell lines were tested for both affinity and capacity of the A77 1726 high-affinity binding site, in addition to the evaluation of behaviour in semi-continuous fermentor culture (results not shown). The histiocytic cell line U937 was chosen as the source of membranes for a purification procedure [3] yielding a single high-affinity A77 1726-binding protein, which was characterized as the mitochondrial enzyme DHO-DH. The purified protein displayed a pI of 9.8, identical to that of the purified mouse spleen enzyme [3], and a molecular mass of 43 kDa (Figure 2), similar to the purified mouse and bovine [11] enzymes, to the value predicted by the human, rat and *Drosophila* gene sequences [12–14] and to the protein immunologically identified in rat liver [15]. It has been claimed that the purified human liver DHO-DH has a molecular mass of approximately 50 kDa [4], although the method of purification and properties of the enzyme have not been published. That human spleen DHO-DH elutes from the cation-exchange column similarly to U937 DHO-DH and is inhibited by A77 1726 analogues with the same potencies (Table 1) suggests that the U937 enzyme is the same as that present in human spleen. The reason for the discrepancy in molecular mass between the human liver enzyme and the enzymes from other sources, including human tissue, is unknown but it cannot be excluded that species- and/or tissue-specific isozymes of DHO-DH exist.

That inhibition of DHO-DH was not an exclusive phenomenon *in vitro* was tested by studying the effect of HR325 on DHO-DH in intact cells. Treatment of stimulated human PBMCs with HR325 for up to 3 days resulted in increases of intracellular DHO and concomitant reductions in intracellular uridine concentrations (Figure 3). These effects are consistent with the high requirement of proliferating T-cells for pyrimidine nucleotides synthesized by the *de novo* pathway and the low-salvage pathway activity in these cells [16].

Supplementation of HR325-treated, PHA/interleukin-2-stimulated human PBMCs with exogenous uridine resulted in a lowered anti-proliferative potency (Figure 4) consistent with an anti-proliferative mechanism reliant on inhibition of DHO-DH. However, complete abrogation was not achieved at higher concentrations of HR325, perhaps indicative of a lower potency anti-proliferative activity. This effect has been noted for A77 1726 in a human T-lymphoblastoma cell line [17] and for the DHO-DH inhibitor brequinar sodium in a murine colon adenocarcinoma cell line [18].

Immunological responses are amplified largely through regulated proliferation of immune cells. Rheumatoid arthritis is an autoimmune disease where parts of the immune system are deranged, notably the proliferative response. That rheumatoid arthritis evolves with hyperproliferative components and sequences makes leflunomide and similar compounds well suited for the treatment of this disease. In fact, the anti-proliferative properties of leflunomide and derived compounds are well in line with the described mechanism of action, inhibition of pyrimidine biosynthesis *de novo*. Sparing of the salvage pathway leads to a targeting of rapidly proliferating cells, i.e. those requiring increased amounts of pyrimidines for DNA synthesis. However, pyrimidines are not only implicated in the synthesis of DNA but also in several other metabolic processes, including RNA synthesis, glycosylation of proteins and lipids, and energy metabolism. It has been shown both *in vitro* and *in vivo* that treatment with leflunomide and analogues results in inhibition of specific immunoglobulin secretion (T. A. Thompson, S. Spinella-Jaegle, E. Francesconi, S. Millet and E. Ruuth, unpublished work). Although it has been proposed that specific immunoglobulins play a role in rheumatoid arthritis, this has not been clearly understood. Nevertheless, a reduction in immunoglobulins could be benefecial. It could also be speculated that inhibition of protein and lipid glycosylation results in changed carbohydrate patterns not only on the outer cell surface but also inside the cell, leading to altered immune and inflammatory responses.

We have purified the high-affinity binding protein of A77 1726 in the human histiocytic lymphoma cell line, U937, and identified it as DHO-DH. The enzyme activity of both U937 cells and human spleen display characteristics typical of the enzymes studied from other mammalian sources. Both A77 1726 and HR325 inhibit DHO-DH activity of purified enzyme, membrane preparations and proliferating cells, thereby reducing the supply of uridine. The pyrimidine requirement of rapidly proliferating cells, but not resting cells, exceeds their salvage capacity [16]. Hence, a *de novo* pyrimidine synthesis inhibitor would have a selective effect on proliferating cells *in vivo*. Since clonal expansion and associated cell proliferation play a key role in immune responses, such an inhibitor would be expected to display characteristics of a selective immunosuppressant. It is important therefore, in light of the current and potential future use of DHO-DH inhibitors as therapeutic agents, that both the anomalous physical characteristics and the kinetic mechanism of the human liver enzyme be fully evaluated.

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