Dihydroorotate Dehydrogenase Is a High Affinity Binding Protein for A77 1726 and Mediator of a Range of Biological Effects of the Immunomodulatory Compound*

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A protein with high affinity (Kd 12 nm) for the immunomodulatory compound A77 1726 has been isolated from mouse spleen and identified as the mitochondrial enzyme dihydroorotate dehydrogenase (EC 1.3.3.1). The purified protein had a pl 9.6–9.8 and a subunit Mₐ of 43,000. Peptides derived from the mouse protein displayed high microsequence similarity to human and rat dihydroorotate dehydrogenase with, respectively, 35 and 39 out of 43 identified amino acids identical. Dihydroorotate dehydrogenase catalyzes the fourth step in de novo pyrimidine biosynthesis. The in vitro antiproliferative effects of A77 1726 are mediated by enzyme inhibition and can be overcome by addition of exogenous uridine. The rank order of potency of A77 1726 and its analogues in binding or enzyme inhibition was similar to that for inhibition of the mouse delayed type hypersensitivity response. It is proposed that inhibition of dihydroorotase dehydrogenase is an in vivo mechanism of action of the A77 1726 class of compounds. This was confirmed using uridine to counteract inhibition of the murine acute graft versus host response.

Leflunomide (N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide, HWA 486) is a novel immunomodulatory and anti-inflammatory compound currently under evaluation in phase III clinical trials for the treatment of rheumatoid arthritis. It has shown dose-dependent clinical efficacy in a 6-month, double-blind, placebo-controlled study on patients with long-standing active arthritis, 81% of whom had failed previous disease-modifying anti-rheumatic drug therapy (1). In addition, it has been shown to be effective in controlling the development of autoimmune disorders and delaying transplant rejection in animals (2–5). Its primary metabolite A77 1726, (Structure 1), has been shown to be effective in inhibiting progression from the G1 to the S phase of the cell cycle (7). Although the biochemical mechanisms by which A77 1726 exerts its effects are unknown, they have been shown to differ from those of other immunosuppressive agents such as corticosteroids, cyclosporin A, rapamycin, or mycophenolic acid (7).

The results presented here describe the purification of a high affinity binding site for A77 1726 and its identification as the mitochondrial enzyme dihydroorotate dehydrogenase. Evidence is presented that identifies the enzyme as a mediator of the in vitro and in vivo effects of the compound (see Structures 1 and 2).

EXPERIMENTAL PROCEDURES

Materials

[2,6-phenyl-3H]A77 1726 (34.3 and 51.4 Ci/mmol, see Structure 1) and [3H]RU35072 (2000 Ci/mmol, Structure 2) were radiolabeled in the Laboratoire de Marquages Isootopiques, Rausse-UCLAF, Romainville, France. Unlabeled test compounds including A77 1726 (see Structure 1) were synthesized in the Chemistry Department, Hoechst Roussel Ltd., Swindon, United Kingdom, except for leflunomide, which was prepared in the Chemistry Department, Hoechst AG, Werk Kalle-Albert, Wiesbaden, Germany, SP-Sepharose HP, PBE138 polybuffer exchange matrix, Pharmalyte pH 8–10.5 and PD-10 desalting columns were purchased from Pharmacia Biotech Inc. Hydroxyapatite (Biolegal HTP) was obtained from Bio-Rad. Trifluoroacetic acid and Biobrene Plus were from Applied Biosystems and endoproteinase Asp-N from Boehringer Mannheim. Coenzyme Q10, dihydroorotic acid, and dichlorophenindophenol (DCIP,1 were obtained from Sigma. Nonyl glucoside was obtained from Sigma or Calbiochem depending on availability or was synthesized in the Chemistry Department of Hoechst Roussel Ltd. using published procedures (8, 9). All other chemicals were obtained from commercial sources.

Methods

All procedures in which native proteins were handled or processed apart from the protein assays themselves were carried out either at 4 °C or on ice.

Preparation of Mouse Spleen Membranes and Soluble Preparations—Crude mitochondrial/microsomal membranes from spleens of CD1 mice (Charles River) were prepared by homogenization and differential centrifugation (10). Homogenization buffer was 25 mM sodium phosphate, 0.25 mM sucrose, 10 μM soybean trypsin inhibitor, 2 μg/ml aprotinin, pepstatin A, and leupeptin, pH 7.4. Slow speed centrifugation was at 470 x g for 10 min, and the membranes were washed by resuspension in homogenization buffer plus 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA before final resuspension in homogenization buffer. For binding studies membranes were prepared in Tris-HCl buffer without the NaCl wash step.

For solubilization, the membranes were diluted to 6 mg/ml protein

1 The abbreviations used are: DCIP, dichlorophenindophenol; DTH, delayed-type hypersensitivity; DHO-DH, dihydroorotate dehydrogenase; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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and mixed with an equal volume of homogenization buffer lacking sucrose but containing 1% nonyl glucoside, 2 mM EDTA, and 2 mM EGTA. The mixture was stirred for 1 h and then centrifuged at 120,000 x g for 60 min. The supernatant was stored at -80 °C for use in binding and preliminary purification studies or filtered through Millipore AP25 polyvinylidene difluoride membranes (Prospin, Applied Biosystems) with 0.1% trifluoroacetic acid at 50 °C and mixed with an equal volume of homogenization buffer lacking KCN, and 20% methanol (three times). The peptide on each membrane was then applied at 1 ml/min to a 4-cm PD-10 column into chromatofocusing elution buffer (1:45 dilution of 300 mM sodium phosphate, pH 7.0, and 500 mM MgCl2 (6 ml), 300 mM sodium phosphate, 0.5% nonyl glucoside, pH 7.0, over a 2-h period. The fraction (60 ml) and eluted at 0.5 ml/min in dialysis buffer with a linear 0–15 mM NaCl gradient (900 ml) containing a 0.1% NaCl plateau (150 ml); remaining protein was removed with 1 nM NaCl (150 ml). The eluted peak of A77 1726 binding activity (100 ml) was stored at -80 °C.

The eluate fraction was thawed and dialyzed against 1 mM sodium phosphate, 0.5% nonyl glucoside, pH 7.0, over a 2-h period. The fraction was then applied at 1 ml/min to a 4 × 1.6 cm hydroxyapatite column previously equilibrated with dialysis buffer. The column was washed (60 ml) and eluted at 0.5 ml/min in dialysis buffer with a linear 0–15 mM MgCl2 gradient (30 ml) and a 15 mM MgCl2 plateau (10 ml). Remaining proteins were removed by successive washes with 0 mM MgCl2 (6 ml), 300 mM sodium phosphate, pH 7.0 (15 ml), and 500 mM sodium phosphate, pH 7.0 (15 ml at room temperature).

The peak of eluted A77 1726 binding activity (12 ml) was desalted on PD-10 columns into chromatofocusing elution buffer (1.45 dilution Pharmalyte pH 8–10.5, 0.5% nonyl glucoside, pH 8.0). A 2.9 × 0.5-cm column of PBE118 polybuffer exchanger, topped with 1 cm of Sephadex G-25 course, was equilibrated with 25 mM triethylamine-acetic acid (0.1%) and subjected to reverse phase chromatography on an RP-300 1 mm × 100-mm column. The column was eluted with a 9-ml linear gradient from 0.1% trifluoroacetic acid to 50% acetonitrile at 0.085% trifluoroacetic acid at 50 μl/min. The peak containing the A77 1726 binding activity was identified by SDS-PAGE, dried, and digested with 0.24 μg of endoproteinase Asp-N for 2 h at 37 °C in 10 mM Tris-HCl, 0.025% SDS, pH 7.5. The mixture was acidified with 1% trifluoroacetic acid and the peptides separated by a repeat of the reverse phase separation procedure.

Of the 23 peptide peaks collected, 5 were dissolved in 40% acetonitrile and applied to Polybrene-treated fiberglass filters. However, no phenylhydantoin-derivatives were released during attempted sequencing. The remaining 18 samples were dissolved in 0.1% trifluoroacetic acid and then 10 mM Tris-HCl, 0.05% SDS and applied to polyvinylidene difluoride membranes (Prospin, Applied Biosystems) with sequential 80-μl washes of 10 mM Tris-HCl, 0.05% SDS, pH 7.5 (twice), and 20% methanol (three times). The peptide on each membrane was sequenced directly in a model 473A microsequencer cartridge (Applied Biosystems).

Photoaffinity Labeling of Mouse Spleen Preparations—Samples were incubated on ice for 1 h in the dark with 0.2–1 nM [3H]RU35072 (see Structure 2) in 25 mM Tris-HCl, 25 mM MgCl2, pH 7.4. High affinity binding was defined by competition with 0.1–10 μM A77 1726 or its analogue HR325 (compound 4 in Table 1). The samples were then irradiated for 5 min at 24 cm with a Mineralite UVC 254 lamp. Non-covalently bound photolabel was removed by dilution with 3 mM HR325 and PD-10 desalting.

Sample Preparation, Electrophoresis, Electrottransfer, and Autoradiography—Sample concentration and discontinuous SDS-PAGE were as described (11, 12). Gels from photoaffinity labeling studies were Coomassie-stained, dried, and autoradiographed using x-ray film (Kodak, X-OMat) and two intensifying screens (DuPont Lightning Plus) at -80 °C for 2–7 days.

Electrophoretic transfer onto Immobilon-P was carried out on a Multiphor II/Novablot semidry system at a constant current of 2 mA/cm² for 60 min in 48 mM Tris-HCl, 39 mM glycine, 0.0375% SDS, 20% methanol or 90 min in a CAPS buffer system (13).

A77 1726-binding assay and data analysis—Membranes or soluble fraction were incubated with 10 or 25 nM [3H]A77 1726 in 10 or 25 mM Tris-HCl, 25 mM MgCl2, pH 7.5. Final assay volumes were 0.5 ml, and nonspecific binding was defined with 3 mM A77 1726. Incubations were allowed to reach equilibrium on ice (2 h for membranes and 16 to 24 h for soluble fractions). Assays were terminated by filtration through Whatman GF/F filters with two 8-ml washes with assay buffer. Filters were dried and counted by liquid scintillation. The [3H]A77 1726 binding characteristics and binding pharmacology of membranes and soluble preparation were determined in competition assays. Competing ligand concentrations increased in ½ log unit intervals, and each concentration was assayed at least in quadruplicate. Binding data were analyzed with the EBDA and LIGAND programs (14). In purification studies high affinity binding was defined as that displasable by 100 nM A77 1726, although this would underestimate the true level slightly.

Dihydroorotate Dehydrogenase Assay—Inhibition of mouse spleen dihydroorotate dehydrogenase activity by test compounds was assessed using a DCIP-linked assay (15). Membranes (0.05 mg of protein) were incubated with 100 μM coenzyme Q10 in 50 mM Tris-HCl, 0.1% Triton X-100, 1 mM KCN, pH 8.0. The reaction was initiated by addition of 500 μM dihydroorotate, and the reduction of DCIP (200 μM) was monitored by loss of absorbance at 650 nm using a 96-well plate reader at 37 °C. Drug concentrations increased in ½ log unit intervals with each concentration tested at least in triplicate. Fractions from purification experiments were assayed in a similar manner, except that the buffer did not contain KCN, and absorbance changes were monitored in a spectrophotometer at 600 nm.

LPS-stimulated Mouse Spleen Cell Proliferation—LPS (12 μg/ml)-stimulated spleen cells (3 × 10⁶ cells/ml) from C57BL/6 mice were incubated for 3 days with increasing A77 1726 concentrations in supplemented RPMI 1640 medium (4 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, and antibiotics, and 10% fetal calf serum) in the presence or absence of uridine (30 μM). Tritiated thymidine incorporation (1 μCi/well) was measured during the final 6 h of the incubation and used as a measure of cell proliferation.

Delayed Type Hypersensitivity (DTH) test—The method used was based on published information (16). Briefly, on day 0 male CD-1 mice (25–30 g, Charles River) were sensitized by subcutaneous injection near the tail base with 1 mg of methylated bovine serum albumin in 0.2 ml of saline/ Freund’s complete adjuvant emulsion. On day 7 mice were challenged by subplantar injection into the hind paw with 0.1 mg of methylated bovine serum albumin in 0.05 ml of saline. The paw edema was expressed as the increase in paw weight after 24 h relative to non-sensitized saline/Freund’s complete adjuvant-injected mice. Drugs were administered orally once per day on days 4–6 and twice on day 7, 1 h before and 6 h after challenge.

Acute Murine Graft versus Host Response (17)—B6C3F1 mice received 1 × 10⁶ C57BL/6 splenocytes intraperitoneally on day 0; controls received RPMI 1640 vehicle. HR325 was administered orally once per day on days 0–3; uridine was administered twice per day subcutaneously (500 mg/kg, at t = 0 and +6 h relative to HR325 administration). Splenomegaly was assessed as spleen weight/body weight ratio on day 10.

Protein Determination—Protein concentrations were generally determined using the bicinchoninic acid method (Pierce). The protein concentration in the chromatofocusing eluate was estimated from area under the curve calculations on the absorbance profile (280 nm) of the step.
RESULTS

Characterization of the Binding Sites in Mouse Spleen Membranes and Soluble Preparations—Binding sites for \( ^{3}H \)A77 1726 in mouse spleen membranes were examined in homologous competition assays. The binding isotherms gave best fits to a three-site model \((p = 0\) versus two-site model) with high \((12 \text{ nm})\), moderate \((3 \mu \text{M})\), and low \((240 \mu \text{M})\) affinity sites detected (Table I, part a). The high affinity site represented \(-75\%\) of the binding curve, whereas the low affinity site was probably saturable nonspecific binding.

The binding characteristics of a series of A77 1726 analogues were tested in competition binding studies. The rank order of potency of the compounds for affinity at the high affinity site was similar to that for the potency of inhibition in the mouse DTH assay (Table I, part b). Compounds 2 and 6 appeared to have higher affinity than expected from their DTH potency; however, the differences were small in view of the in vitro/s in vivo nature of the comparison.

The high affinity binding site was solubilized with 0.5% nonyl glucoside (45% yield; Table I, part a). The site showed maintained affinity for A77 1726. The moderate affinity site was not solubilized in a detectable form.

Purification of the High Affinity Binding Site—Sequential chromatography on SP-Sepharose HP, hydroxyapatite, and chromatofocusing columns afforded high affinity binding site yields of 65%, 41%, and 72% step, respectively. Additional losses were observed on the dialysis and desalting steps used to prepare eluates for further chromatography, the overall yield of the procedure was thus 14% with 1988-fold purification (Table II). The high affinity site started to elute from the chromatofocusing column at pH 9.8, consistent with a preliminary experiment where the site eluted between pH 9.79 and 9.58.

Characterization of the Purified High Affinity Binding Site—The chromatofocusing eluate contained a single band of Mr 43,000 on SDS-PAGE following electrophoresis to an Immobilon-P membrane and Coomassie staining (Fig. 1, lane 1). Silver staining was not attempted as an earlier experiment indicated that the Mr 43,000 band stained very poorly. The Mr 43,000 protein was identified as the high affinity site in photoaffinity labeling studies using the iodoazido compound (Structure 2) on soluble preparation and eluates from small scale purification experiments (Fig. 1, lanes 2-5). Additional studies on a single-step chromatofocusing eluate and an SP-Sepharose-hydroxyapatite eluate, prepared with phosphate rather than magnesium gradient elution, showed complete and almost complete protection, respectively, of the Mr 43,000 band by 100 \( \mu \text{M} \) HR325 (data not shown).

An attempt at obtaining N-terminal sequence from the Mr 43,000 band was unsuccessful. In order to obtain internal sequence, the purification procedure was scaled up 2-fold, yielding 210 pmol of high affinity site, 1% of which produced a faint single band of Mr 43,000 on SDS-PAGE and Coomassie staining. The eluate was subjected to reverse phase high performance liquid chromatography to remove detergent-derived non-protein contaminants, which would prevent peptide detection, together with any minor contaminating proteins not detected on the Coomassie-stained gel. The Mr 43,000 protein was subjected to endopeptidase digestion (Asp-N), with the resulting peptides separated by an additional round of reverse phase chromatography. Sequence data was obtained for 7 of the 18 peptides applied to polyvinylidene difluoride membranes (Fig. 2). A search of the Swissprot data bank revealed that only human dihydroorotate dehydrogenase (DHO-DH) had amino acid sequence similarity with all the sequenced peptides. For the five peptides that could be unambiguously aligned with the human sequence, 35 out of 43 assigned amino acids were identical (Fig. 2). A greater degree of similarity was observed when the peptides were compared with rat DHO-DH sequence (EMBL data base, accession no. X80778) with 39 out of 43 residues identical. The remaining two pentapeptides could be

| Membrane binding affinity (Kd) | Binding capacity (Bmax: pmol/mg) |
|-------------------------------|---------------------------------|
| site 1 | site 2 | site 3 | site 1 | site 2 | site 3 |
| Membranes | 1223 | 321 | 202110 | 3.82 | 0.68 | 7.00e+3000 |
| Soluble | 26 | - | 90 | 2.4 | - | 1000 |

| No. | R1 | R2 | R3 | R4 | Binding affinity (Kd) | % inhibition | Mouse spleen membrane |
|-----|----|----|----|----|---------------------|-------------|----------------------|
| 1   | CF3 | H | CN | - | 9  | 1  | 75 (30)** | DHO-DH |
| 2   | CF3 | H | CN | - | 15 | 2  | 54**  | 86 ± 16 |
| 3   | CF3 | H | CN | CH3 | 18 | 4  | 84, 62, 75, 99f(30)** | 91 ± 12 |
| 4   | CF3 | CH3 | CN | - | 20 | 1  | 81 (30)** | 57 ± 10 |
| 5   | CF3 | H | CN | - | 20 | 1  | 85 (30)** | 173 ± 6 |
| 6   | CF3 | H | CN | - | 32 | 2  | 64*, 47 | 92 ± 8 |
| 7   | H   | CF3 | CN | - | 108| 14 | 85** | 579 ± 17 |
| 8   | CH3CO | H | CN | - | 383| 10 | 32 & 12 | 1400 ± 200 |
| 9   | CF3 | H | NO2 | - | 11000 | 2 | 19 | >100000 |
| 10  | OH | H | CN | - | 3000 | >30 | -11 | >50000 |
| 11  | CF3 | H | CN | - | >100000 | >100 | 10 | >100000 |

Table I: \( ^{3}H \)A77 1726 binding characteristics of membrane and soluble preparations and comparison of binding pharmacology with potency in DTH and DHO-DH assays
aligned with several possible sequences within the human and rat proteins, with 3 of the 5 residues being identical in each case.

Further evidence supporting the identity of the high affinity site as DHO-DH—Fractions from a series of purification experiments were analyzed for DHO-DH using the DCIP assay. In all cases DHO-DH activity and high affinity binding activity co-purified (data not shown).

The reversible nature of the binding interaction before photolysis was further established in an experiment in which membranes were incubated for 2 h with 235 pmol photoaffinity ligand. Although this concentration should occupy >85% of the high affinity sites, more than 98% of the original binding in the membranes was detected following dilution of the membranes into a [3H]A77 1726 binding assay (5 nM final photoaffinity ligand concentration). Irreversible binding of the radioactive photolabel to mouse spleen membranes was not observed in the absence of photolysis (data not shown). Molecular size markers are indicated in full for 1 and 2, by position for 3 and by position for M, 45,000 and 36,000 markers only for 4 and 5. The M, 43,000 bands in the different gels are aligned.

**DISCUSSION**

The aim of the current study was to isolate and characterize the target protein that mediates the effects of A77 1726, the active metabolite of leflunomide. A potential target protein was identified in mouse spleen membranes using a radioligand binding approach (Table I). Although the membranes carried at least three binding sites, the high affinity site (K_d 12 nm) was of most interest as its binding pharmacology for A77 1726 analogues was qualitatively similar to that for the mouse DTH response in vivo (Table I). The DTH assay was used as a primary screen to detect active compounds; as such, dose ranges were limited, preventing quantitative correlation between binding and potency. However, the qualitative relationship, which extended to over 70 compounds, supported the role of the high affinity site in drug action in vivo.

The purified site was unequivocally identified as DHO-DH by a series of structural and functional criteria. There was high microsequence identity between human enzyme (81% Ref. 19) or rat enzyme (91% EMBL data base) and pure peptides derived from the high affinity site (Fig. 2). In addition, the relative molecular weight of 43,000 for the site on SDS-PAGE (Fig. 1) was close to that of the bovine liver enzyme (M, 42,000; Ref. 20) and that predicted for human DHO-DH (43.0 kDa; Ref. 19). The weak silver staining of the mouse protein is consistent with a protein lacking cysteine residues, as is the case with the human enzyme (19). A77 1726 binding affinity of DHO-DH—

**TABLE II**

### Purification of the mouse spleen high affinity [3H]A77 1726 binding site

| Protein                  | Total Yield | Specific activity | Purification |
|--------------------------|-------------|------------------|--------------|
| Nonyl glucoside-solubilised | 73 | 100 | 1.64 | 1 |
| S-Sepharose               | 73 | 100 | 1.64 | 1 |
| Hydroxylapatite           | 73 | 100 | 1.64 | 1 |
| Chromatofocusing          | 73 | 100 | 1.64 | 1 |

**Fig. 1. Representative profiles of selected fractions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** All samples were separated on discontinuous gels comprising a 5% stacking gel and 10% resolving gel (except for the autoradiograph of the SP-Sepharose eluate where a 4% stacking gel/12% resolving gel was used). Chromatofocusing eluate, electrophoresed under an immobilized-P membrane and Coomassie-stained (lane 1). Autoradiographs of photoaffinity-labeled solubled preparation (lane 2), SP-Sepharose eluate (lane 3), hydroxylapatite eluate (lane 4), and chromatofocusing eluate (lane 5). In each case, for photoaffinity labeling were incubated in the dark for 1 h with the [125I]iodoazido ligand (see Structure 2) prior to photolysis (lanes 2a, 3a, 4a, and 5a). Parallel incubations, in which the photoaffinity ligand had a K_d 26 nM for the mouse (Fig. 3).

**Fig. 2.** Autoradiographs of photoaffinity-labeled fractions for photoaffinity labeling were incubated in the dark for 1 h with the [125I]iodoazido ligand (see Structure 2) prior to photolysis (lanes 2a, 3a, 4a, and 5a). Parallel incubations, in which the photoaffinity ligand had a K_d 26 nM for the mouse (Fig. 3).
Effect of uridine on antiproliferative activity of A77 1726. Tritiated thymidine incorporation in mouse spleen cells stimulated with LPS and incubated for 3 days in the presence of a range of A77 1726 concentrations in the presence (□) or absence (▲) of 30 μM uridine (see “Experimental Procedures” for detail). Control wells were cultured alone (177 cpm) or with LPS (59,510 cpm), LPS + 30 μM uridine (54,604 cpm), LPS + Me2SO (54,300 cpm), and LPS + Me2SO + 30 μM uridine (47,661 cpm).

The possibility that the A77 1726 class of compounds may have additional effects in vivo or in vitro cannot be ruled out by the current studies. However, some of the previously reported effects of long-term treatment of cells with the compounds could be secondary to pyrimidine depletion. For example, inhibition of epidermal growth factor-dependent tyrosine kinase activity in intact cells (24) was demonstrated after a 4-day treatment with A77 1726, whereas a 30-min treatment had no effect (7). However, proof that this effect reflects pyrimidine nucleotide depletion awaits uridine reversal studies.

In conclusion, the A77 1726 high affinity binding protein in murine spleen membranes, proposed as a putative target for A77 1726 action, has been isolated and identified as dihydroorotate dehydrogenase. The enzyme catalyzes the fourth step in de novo pyrimidine biosynthesis and its inhibition accounts for the antiproliferative effects of the compounds in vitro and some of the in vivo effects of the compounds.

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