Biochemical Pharmacology, Vol. 52, pp. 527–534, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII 0006-2952(96)00303-6

Two Activities of the Immunosuppressive Metabolite of Leflunomide, A77 1726

INHIBITION OF PYRIMIDINE NUCLEOTIDE SYNTHESIS AND PROTEIN TYROSINE PHOSPHORYLATION

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ABSTRACT. Previous studies have demonstrated that the active metabolite of leflunomide, A77 1726 [*N*-(4-trifluoromethylphenyl-2-cyano-3-hydroxycrotoamide)], is capable of inhibiting the activities of tyrosine kinases and dihydroorotate dehydrogenase (DHO-DHase). In the present study, we define the relative contribution of these activities to the ability of A77 1726 to inhibit proliferation of the murine leukemia cell line LSTRA. A77 1726 inhibited LSTRA cell growth and proliferation ($IC_{50} \approx 10$–30 μM); this inhibition, however, could be reversed by the addition of exogenous uridine, suggesting that the anti-proliferative activity of A77 1726 may be due to inhibition of *de novo* pyrimidine nucleotide synthesis. Quantitation of nucleotide levels revealed that A77 1726, at an IC_{50} of about 10 μM, selectively inhibited pyrimidine nucleotide but not purine nucleotide synthesis. *In vitro* enzyme assays confirmed that A77 1726 directly inhibited the activity of DHO-DHase, the fourth enzyme in the *de novo* pathway of pyrimidine nucleotide synthesis ($IC_{50} \approx 220$ nM). LSTRA cells overexpress $p56^{lck}$ and have elevated levels of tyrosine phosphorylated intracellular proteins. A77 1726 reduced the intracellular levels of tyrosine phosphorylated proteins with relatively high IC_{50} values ranging from 50 to 100 μM. A77 1726 also inhibited $p56^{lck}$ activity in LSTRA membrane preparation and immunoprecipitates; the IC_{50} values for inhibition of immunoprecipitated $p56^{lck}$ autophosphorylation and exogenous substrate histone 2B were 80 and 40 μM, respectively. The anti-tyrosine phosphorylation activity of A77 1726 was not affected by uridine. These studies therefore demonstrate the two activities of A77 1726: inhibition of pyrimidine nucleotide synthesis and interference with tyrosine phosphorylation. BIOCHEM PHARMACOL 52;4:527–534, 1996.

KEY WORDS. LSTRA cells; A77 1726; tyrosine kinase inhibitor; inhibition of dihydroorotate dehydrogenase; brequinar sodium; nucleotides

Leflunomide [*N*-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486] is an isoxazol derivative that is reported to express potent immunomodulatory and anti-inflammatory activities. Leflunomide shares no apparent structural relationship with the existing immunosuppressive agents, and the mechanism of immunosuppression is the subject of active investigation. The primary metabolite of leflunomide, A77 1726 [*N*-(4-trifluoromethylphenyl-2-cyano-3-hydroxycrotoamide)], has been reported to mediate immunosuppressive activities similar to the parent compound. Early *in vitro* studies with A77 1726 revealed that A77 1726 suppressed the proliferation of human peripheral T lymphocytes stimulated with allogeneic lymphocytes in a one-way mixed lymphocyte reaction (MLR), and also when stimulated with anti-CD3 plus phorbol 12-myristate 13-acetate (PMA) or anti-CD28 plus PMA [1, 2]. We have reported previously that A77 1726 is capable of inhibiting anti-CD3-stimulated tyrosine phosphorylation in Jurkat T cells, and subsequent phospholipase C-γl activation, $[Ca^{2+}]_i$ flux as well as IL-2[‖] production and IL-2 receptor expression [3]. In addition, we also reported that A77 1726 is able to inhibit the tyrosine phosphorylation and proliferation induced by IL-2 in an IL-2-dependent cell, CTLL-4 [3].

Recently, it was reported that the anti-proliferative activity of A77 1726 can be reversed by the addition of uri-

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Received 5 January 1996; accepted 11 March 1996.

‖ *Abbreviations:* DHO-DHase, dihydroorotate dehydrogenase; DCIP, 2,6-dichlorophenol-indophenol; IL-2, interleukin-2; ECL, enhanced chemiluminescence; PTK, protein tyrosine kinase; and BQR, brequinar sodium.

dine and/or cytidine, suggesting that A77 1726 may interfere with *de novo* pyrimidine nucleotide synthesis [4–6]. It was further reported that inhibition of *de novo* pyrimidine nucleotide synthesis was by direct inhibition of the mitochondrial enzyme DHO-DHase [7, 8], which catalyzes the fourth step in *de novo* pyrimidine nucleotide biosynthesis.

LSTRA is a leukemia T cell line that overexpresses the *src* family kinase, $p56^{lck}$. The enhanced expression of $p56^{lck}$ in LSTRA cells is the result of retroviral promoter insertion between the proximal and distal promoters of $p56^{lck}$ gene [9]. Overexpression of $p56^{lck}$ results in the elevated tyrosine phosphorylation of intracellular proteins, and with disregulated cell proliferation and lymphoid malignancy [9–11]. In this report, we characterize the relative contributions of pyrimidine nucleotide synthesis and tyrosine kinase inhibition to the ability of A77 1726 to inhibit LSTRA cells proliferation.

MATERIALS AND METHODS

Cells

The murine leukemia cell line LSTRA was a gift from Dr. Tamara R. Hurley (Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, CA). Cells were grown in complete RPMI 1640 medium containing 15% fetal bovine serum and 50 μM 2-mercaptoethanol.

Reagents

The primary metabolite of leflunomide, A77 1726, and BQR were gifts from Dr. Robert Bartlett (Hoechst AG Werk Albert, Wiesbaden, Germany). Stock solutions (10 mM) of A77 1726, dissolved in distilled water, were stored at 4° for up to 2 weeks. BQR was dissolved in 100% ethanol. Genistein (Gibco, Grand Island, NY) was made up at 1 mg/mL in DMSO and stored at −20°. Rabbit anti-$p56^{lck}$ antiserum and anti-phosphotyrosine mAb 4G10 were purchased from UBI (Lake Placid, NY). Histone 2B and Pansorbin were purchased from Calbiochem (San Diego, CA). ATP, CTP, UTP, GTP, dihydroorotic acid, coenzyme Q10, and DCIP were purchased from Sigma (St. Louis, MO).

In vitro *Tyrosine Kinase Assay of LSTRA Membrane*

LSTRA cell membranes were prepared following protocols established by Lin *et al.* [12] and stored at −70° prior to use. LSTRA membrane protein (2 μg) was preincubated with various concentrations of A77 1726 at 4° for 10 min, then 2 μg of histone 2B was added, and the mixture was incubated at 4° for another 10 min. [^{32}P-γ]ATP (10 μCi) was added to initiate the tyrosine kinase reaction, which was performed in a total volume of 50 μL PTK buffer (50 mM HEPES, pH 7.4; 10 mM $MgCl_2$; and 10 mM $MnCl_2$). The kinase reaction was performed at room temperature for 10 min, and then 50 μL of 2× sample buffer [20% glycerol (v/v); 2% SDS; 25% 4× stacking gel buffer; 5% β-mercaptoethanol; 0.0025% bromophenol blue] was added to stop the reaction. The mixture was separated on a 12% SDS polyacrylamide gel, the gel was dried, and protein phosphorylation was visualized by exposure to X-Omat film (Sigma).

Immunocomplex Tyrosine Kinase Assay

$p56^{lck}$ was immunoprecipitated with anti-$p56^{lck}$ antiserum as previously described [13]. Briefly, 1×10^7 LSTRA cells were pelleted and then lysed in 1 mL of Brij 96 buffer [20 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1 mM phenylmethylsulfonyl fluoride; 1% Brij 96 (w/v)]. The lysates were prepared, and then precleared by incubation with 100 μL Pansorbin and sedimentation. The precleared cell lysates were incubated with 5 μL of anti-$p56^{lck}$ or normal rabbit serum (NRS) at 4° for 2 hr. $P56^{lck}$ was immunoprecipitated with 30 μL protein A agarose beads (Oncogene Science, Uniondale, NY), washed three times with Brij 96 buffer, and then resuspended in PTK buffer. Aliquots of immunoprecipitated $p56^{lck}$ were transferred to each tube containing various concentrations of A77 1726 or genistein. Tyrosine kinase assay was performed essentially as described for LSTRA cell membranes above. To examine the effect of uridine, immunoprecipitated $p56^{lck}$ was preincubated with A77 1726 in the presence of various concentrations of uridine; then the tyrosine kinase assay was performed as described above.

Western Blot Analyses

LSTRA cells (2×10^6) were preincubated with A77 1726 in 2 mL RPMI 1640 containing 15% fetal bovine serum at 37° for 6 hr in a 5% CO_2 incubator. Then cells were lysed in 100 μL of Nonidet P-40 (NP-40) buffer [50 mM Tris-HCl, pH 8.0; 2 mM EDTA; 0.15 M NaCl; 1% NP-40 (v/v); 200 μM Na_3 VO_4; 1 mM phenylmethylsulfonyl fluoride; and 10 μg/mL of each aprotinin and leupeptin]. The protein concentration of each sample was quantitated by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Fifty micrograms of each lysate was separated on a 10% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane. Protein tyrosine phosphorylation was detected by anti-phosphotyrosine mAb (4G10) and ECL techniques as recommended by the manufacturer (Amersham, Arlington Heights, IL). To determine whether the inhibition of protein tyrosine phosphorylation by A77 1726 is time-dependent, 2×10^6 LSTRA cells were incubated with 100 μM A77 1726 for various times. The lysates were prepared, and protein tyrosine phosphorylation was examined as described above.

Cell Proliferation Assay

LSTRA cells (1×10^4) were seeded in 96-well flat-bottom microtiter plates in complete RPMI 1640 medium (15% fetal bovine serum and 50 μM 2-mercaptoethanol) in the

presence of various concentrations of A77 1726 for 0, 24, 48, or 72 hr and then pulsed with 1 μCi [^{3}H]thymidine (6.7 Ci/mmol)/well for 2 hr. The cells were harvested by an automatic 96-well harvester (Harvester 96, Tomtec, Orange, CT), and the [^{3}H]thymidine uptake was quantitated on a scintillation counter (1205 Betaplate™, LKB, Gaithersburg, MD). To examine the effect of uridine on the inhibition of LSTRA cell proliferation by A77 1726, 1 × 10^4 LSTRA cells were seeded in 96-well flat-bottom microplates in the presence of A77 1726 and uridine. After 24 hr, the cells were pulsed and harvested as described above.

Cell Growth Assay

LSTRA cells (5 × 10^4) were seeded in 24-well plastic tissue culture plates in 1 mL of complete RPMI 1640 medium (15% fetal bovine serum and 50 μM 2-mercaptoethanol) with various concentrations of A77 1726. Cell numbers in each well were counted at various times, and cell viability was monitored by trypan blue exclusion staining.

Quantitation of Tyrosine Phosphorylation

The exposed X-Omat films from the *in vitro* tyrosine kinase assays or the phosphotyrosine proteins detected on western blots were scanned in an LKB densitometer (2202 Ultrascan Laser Densitometer, LKB). The peaks corresponding to the bands of interest were integrated to determine the relative amounts of phosphorylation.

In vitro *DHO-DHase Assay*

Mitochondria were prepared by homogenization of LSTRA cells and differential centrifugation as described elsewhere [14]. The measurement of the DHO-DHase activity in mitochondria was performed as previously described [15]. Briefly, mitochondria at the final protein concentration of 0.75 mg/mL were preincubated with various concentrations of A77 1726 at 37° for 30 min in a buffer containing 0.1% Triton X-100, 0.2 mM DCIP, 0.1 mM coenzyme Q10, 1 mM KCN, and 48 mM Tris–HCl, pH 8.0, in a 96-well microplate. The DHO-DHase reaction was initiated by the addition of 500 μM dihydroorotic acid substrate and continued for 30 min at 37° (within 60 min, the rate of the enzymatic activity of DHO-DHase is in linear range under the conditions used here). The wells without dihydroorotic acid were used for background values. The enzymatic activity was monitored by the decrease of OD_{600} nm values on an ELISA plate reader (Bio-Rad, Richmond, CA).

HPLC Analyses of the Intracellular Nucleotide Pool

LSTRA cells grown to a density of 0.5–1 × 10^6 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% horse serum were incubated with various concentrations of A77 1726 or BQR for 18 hr. Nucleotides were extracted with 4% perchloric acid, neutralized with 5 N KOH as described [16], and then analyzed in a Waters HPLC system with a 616 pump, a 600S gradient controller, a 717 plus autosampler and a 996 PDA detector (Milford, MA). The separation was achieved by a linear gradient elution of potassium phosphate buffer, pH 4.5 (10–500 mM) on a Whatman strong anion exchange column, Partisil 10 SAX (Alltech, Deerfield, IL). The corresponding peaks of four nucleotides were integrated, and the concentrations were calculated based on a standard curve.

RESULTS

Inhibitory Effects of A77 1726 *on LSTRA Cell Growth and Proliferation*

Consistent with previous studies documenting antiproliferative activity [1, 2], A77 1726 was able to inhibit the growth and proliferation of LSTRA cells, a murine leukemia cell line overexpressing $p56^{lck}$. As shown in Fig. 1, A and B, A77 1726 at concentrations ranging from 10 to 50 μM inhibited cell growth in both a concentration- and time-dependent manner. The half-maximal inhibition (IC_{50}) value of A77 1726 to inhibit cell proliferation was approximately 30 μM as measured by changes in cell number, and was approximately 10–20 μM as measured by changes in thymidine incorporation. A77 1726 at concentrations of ≤150 μM did not decrease cell viability as determined by trypan blue exclusion staining over the time-course of the experiment (data not shown).

Inhibitory Effect of A77 1726 *on* $p56^{lck}$ *Tyrosine Kinase in LSTRA Cell Membrane*

A retroviral gene insertion between distal and proximal promoters of $p56^{lck}$ gene results in increased gene transcription, and, subsequently, a 40-fold increase in $p56^{lck}$ expression in LSTRA cells [13]. Since the activation of protein tyrosine kinases plays a pivotal role in the induction of cell proliferation, we hypothesized that the ability of A77 1726 to inhibit cell proliferation may be a consequence of inhibition of $p56^{lck}$. We first examined the inhibitory effect of A77 1726 on LSTRA cell membrane-associated kinase using an *in vitro* kinase assay. As shown in Fig. 2A, A77 1726 inhibited the phosphorylation of two dominant membrane-associated proteins of 56 and 120 kDa, as well as the phosphorylation of the 14 kDa exogenous substrate, histone 2B. The 56 kDa protein was identified as $p56^{lck}$ by immunoprecipitation with anti-$p56^{lck}$ serum (data not shown); the identity of the 120 kDa protein was not determined. Half-maximal inhibition concentrations of A77 1726 for the tyrosine phosphorylation of $p56^{lck}$ and p120 protein, calculated by densitometric analyses, were approximately 145 μM, while the IC_{50} value for histone 2B was 70 μM, as determined by densitometric analysis.

Protein phosphorylation using the LSTRA membrane kinase assay could reflect phosphorylation by serine or threonine kinases as well as tyrosine kinases. Therefore the immune-complex tyrosine kinase assay was performed using

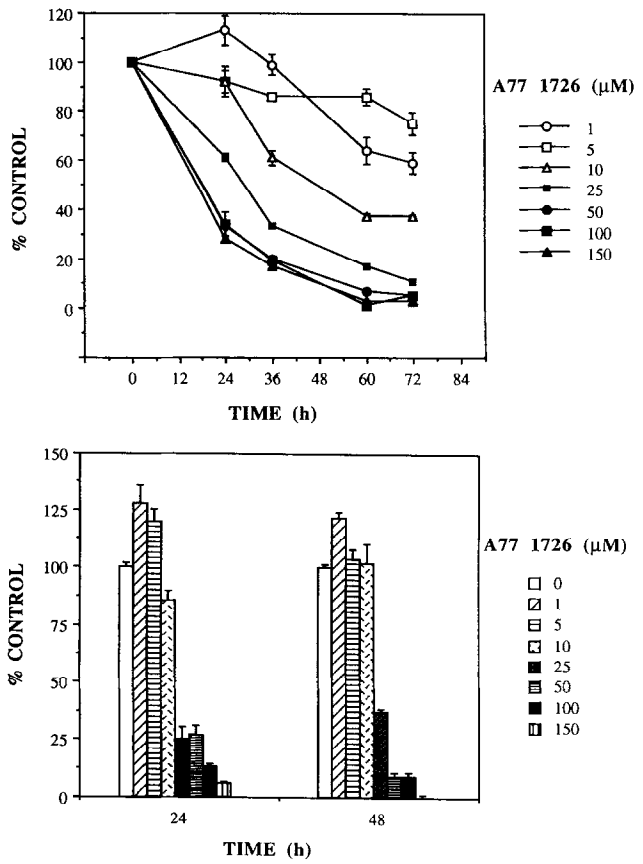

FIG. 1. **Inhibitory effects of A77 1726 on LSTRA cell growth and proliferation. (A) Inhibition of LSTRA cell growth by A77 1726. LSTRA cells (5×10^4) were aliquoted into 24-well plates (Costar) in the presence of the indicated concentrations of A77 1726. Data from one of three independent experiments are presented as percent of control = (number of cells in A77 1726-treated wells – number of cells at the start of the experiment)/(number of cells in untreated wells – number of cells at the start of the experiment) × 100 ± SEM, where 100% represents the number of cells in untreated wells. (B) Inhibition of LSTRA cell proliferation by A77 1726. LSTRA cells (1×10^4) were seeded, in triplicate, into each well of a 96-well microtiter plate in the absence or the presence of various concentrations of A77 1726. After 24, or 48 hr, DNA synthesis was measured by pulsing the cells with [^{3}H]thymidine for 2 hr. [^{3}H]Thymidine uptake was quantitated in a scintillation counter, and data from one of five similar experiments are presented as percent of control of ± SEM.**

immunoprecipitated p56^{lck} from LSTRA cell lysates. As shown in Fig. 2B, A77 1726 inhibited p56^{lck} autophosphorylation at an IC_{50} of 80 μM and phosphorylation of histone 2B at an IC_{50} of 40 μM. Genistein, a well-defined PTK inhibitor included in this experiment as a positive control, had a similar capacity to inhibit p56^{lck} autophosphorylation and phosphorylation of histone 2B (Fig. 2C).

Inhibitory Effect of A77 1726 *on Protein Tyrosine Phosphorylation in LSTRA Cells*

The observations that A77 1726 inhibited p56^{lck} activity *in vitro* led to the prediction that A77 1726 would also be able

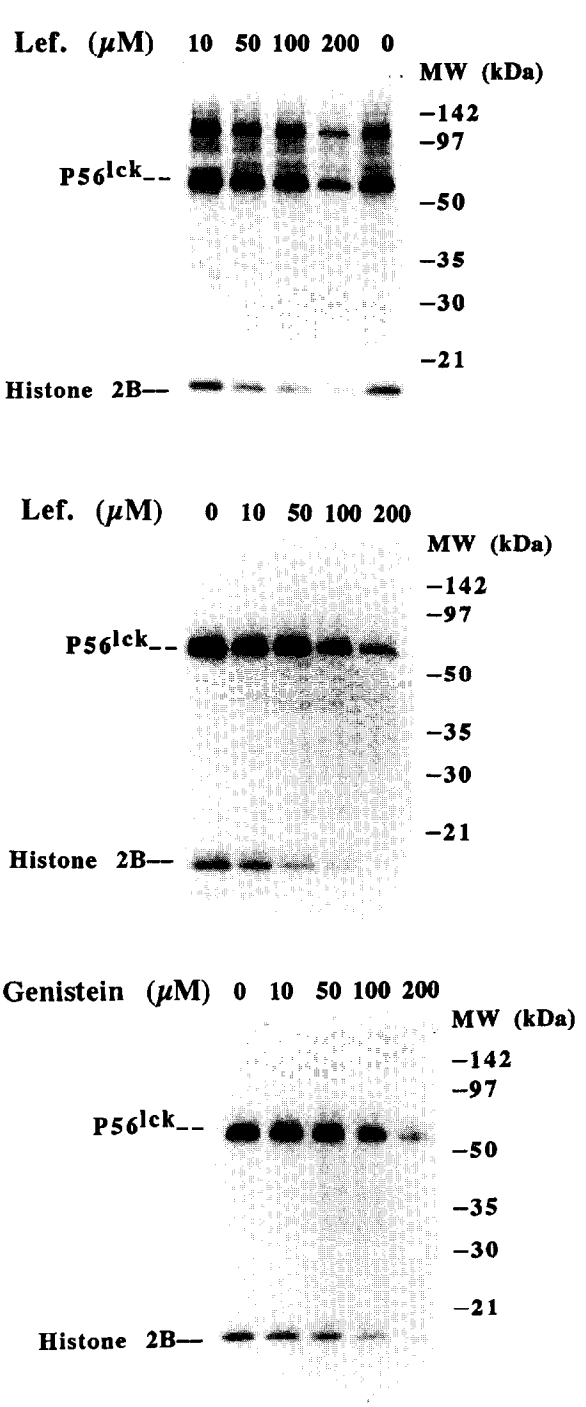

FIG. 2. **Inhibitory effects of A77 1726 and genistein on p56^{lck} activity in *in vitro* kinase assays. (A) Inhibition of p56^{lck} tyrosine kinase activity in LSTRA cell membranes by A77 1726. LSTRA cell membranes were prepared and tyrosine kinase assays were performed as described in Materials and Methods, in the presence of the indicated concentrations of A77 1726. (B) Inhibition of immunoprecipitated p56^{lck} activity by A77 1726. p56^{lck} was immunoprecipitated, and *in vitro* tyrosine kinase assays were performed in the presence of the indicated concentrations of A77 1726 as described in Materials and Methods. (C) Inhibition of p56^{lck} activity by genistein in immune-complex tyrosine kinase assays. Lef. stands for A77 1726.**

to inhibit protein tyrosine phosphorylation in cultured LSTRA cells. LSTRA cells were incubated with various concentrations of A77 1726 for 6 hr, and then tyrosine phosphorylation of cellular substrates was analyzed by western blot. As shown in Fig. 3A, A77 1726 differentially inhibited protein tyrosine phosphorylation of various substrates in a concentration-dependent manner. However, tyrosine phosphorylation of the proteins around 56 and 90 kDa was resistant to A77 1726. The IC_{50} values for inhibition of tyrosine phosphorylation of the rest of the substrates (85 and 120 kDa) ranged between 50 and 100 μM.

Inhibition of protein tyrosine phosphorylation by A77 1726 was time dependent (Fig. 3B). Incubation of LSTRA cells with 100 μM A77 1726 for 10 min did not reduce tyrosine phosphorylation of all intracellular proteins significantly, while incubation for ≥6 hr achieved maximal inhibition for many proteins (85, 90, 100, and 120 kDa). However, phosphorylation of some intracellular proteins (approximately 50 and 56 kDa) was not reduced despite treatment for up to 24 hr with A77 1726.

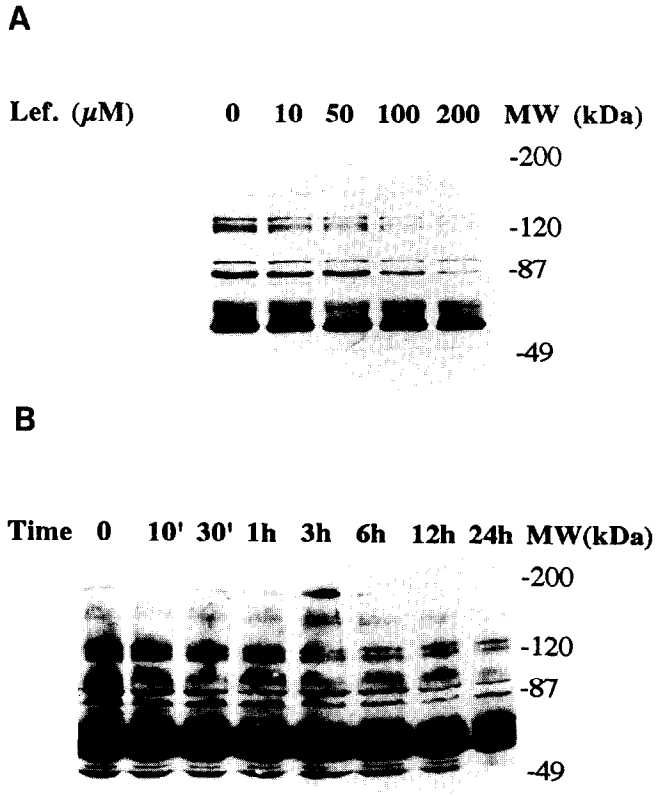

FIG. 3. Inhibition of protein tyrosine phosphorylation in LSTRA cells by A77 1726. (A) LSTRA cells were incubated with the indicated concentrations of A77 1726 for 6 hr at 37°; then western blotting was performed to monitor the levels of protein tyrosine phosphorylation as described in Materials and Methods. (B) Time-dependence of inhibition of protein tyrosine phosphorylation in LSTRA cells by A77 1726. LSTRA cells (2 × 10^6) were incubated with 100 μM A77 1726 for the indicated times, cell lysates were prepared, and the levels of protein tyrosine phosphorylation were determined by western blotting.

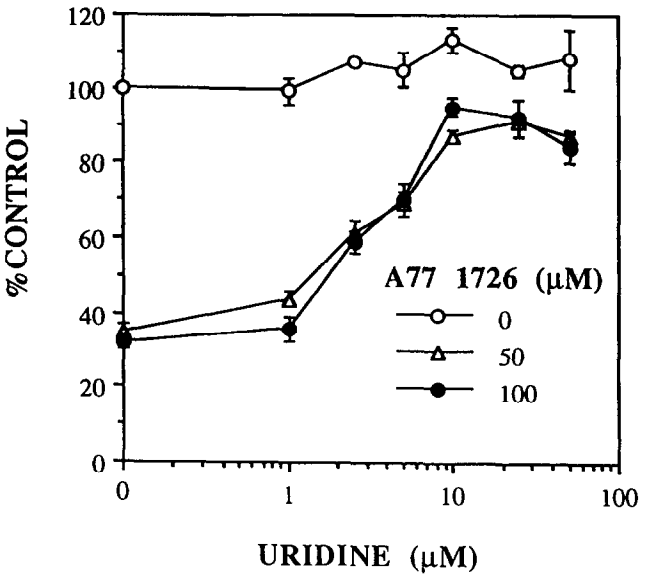

FIG. 4. Ability of uridine to reverse inhibition of LSTRA cell proliferation by A77 1726. LSTRA cells were seeded in a 96-well microplate and incubated with 50 or 100 μM A77 1726 in the absence or presence of various concentrations of uridine. After 24 hr, the cells were pulsed with 1 μCi [^{3}H]thymidine for 2 hr, and then the cells were harvested. [^{3}H]Thymidine uptake was quantitated on a scintillation counter, and the results are presented as mean percent control from 4 independent experiments ± SEM. In the absence of A77 1726 and uridine, the cpm of [^{3}H]thymidine-labeled LSTRA cells was 68,900 ± 1,500.

Reversal of the A77 1726*-Mediated Anti-proliferative Effect by Uridine*

Since the IC_{50} values required to inhibit cellular tyrosine phosphorylation were higher than those required to inhibit cell growth and proliferation, the possibility existed that A77 1726 may have another effect(s) on LSTRA cells. It has been observed that uridine interfered with the inhibitory effect of A77 1726 on human lymphocyte proliferation [4–6]. We tested here whether uridine could antagonize the inhibitory effect of A77 1726 on LSTRA cell proliferation. LSTRA cells were incubated with A77 1726 in the presence of various concentrations of uridine for 24 hr; then DNA synthesis was quantitated by [^{3}H]thymidine incorporation. As shown in Fig. 4, uridine reversed the inhibition of LSTRA cell proliferation by either 100 or 50 μM A77 1726 in a concentration-dependent manner. Uridine at a concentration of 10 μM maximally reversed inhibition of proliferation mediated by 100 or 50 μM A77 1726, whereas uridine alone increased LSTRA cell proliferation by 5–15%. These results suggest that uridine can reverse the anti-proliferative effect of A77 1726 in a non-competitive manner.

Inability of Uridine to Reverse the Inhibition of Protein Tyrosine Phosphorylation

We proposed previously that A77 1726 functions as a tyrosine kinase inhibitor to block T lymphocyte proliferation

[3]. Our observation that uridine antagonizes the ability of A77 1726 to inhibit LSTRA cell proliferation led us to test whether uridine also interfered with inhibition of protein tyrosine phosphorylation by $p56^{lck}$ activity in an *in vitro* tyrosine kinase assay. Shown in Fig. 5A, A77 1726 at 100 μM inhibited the autophosphorylation of $p56^{lck}$ and the phosphorylation of histone 2B by 48 and 88%, respectively (compare lanes 2 and 1); uridine at 5–50 μM, the concentrations that efficiently reversed the inhibition of LSTRA cell proliferation, did not antagonize the inhibition of $p56^{lck}$ autophosphorylation or the phosphorylation of histone 2B. In addition, uridine at a concentration of 10 μM did not antagonize the ability of A77 1726 to reduce the levels of protein tyrosine phosphorylation in LSTRA cells (compare lane 2 with lane 3) (Fig. 5B). It appears that uridine at 25 or 50 μM slightly restored tyrosine phosphorylation of 180 and 120 kDa proteins (compare lanes 4 and 5 with lane 2). However, uridine alone at 10–50 μM also slightly increased tyrosine phosphorylation (compare lanes 6–8 with lane 1); the reason for the effect of uridine is not clear. Therefore, the slight increase of tyrosine phosphorylation seen in lanes 4 and 5 could be the non-specific stimulatory effect of uridine rather than the antagonistic effect of uridine. These observations indicated that reversal of inhibition of LSTRA cell proliferation by uridine is not due to restoration of intracellular tyrosine phosphorylation and suggest that A77 1726 has two effects in LSTRA cells–inhibition of tyrosine kinase activity and inhibition of pyrimidine nucleotide synthesis.

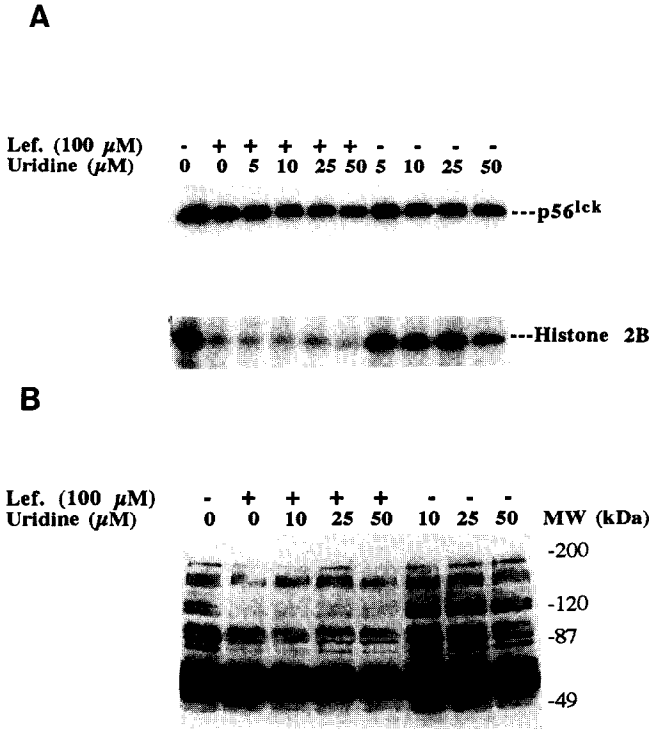

FIG. 5. Inability of uridine to reverse the inhibition of protein phosphorylation by A77 1726. (A) Immunoprecipitated $p56^{lck}$ was incubated with 100 μM A77 1726 in the absence or presence of various concentrations of uridine for 10 min; then the tyrosine kinase reaction was performed as described in Materials and Methods. (B) LSTRA cells were incubated with 100 μM A77 1726 in the absence or presence of various concentrations of uridine for 6 hr. The levels of protein tyrosine phosphorylation were determined by western blotting. Lef. stands for A77 1726.

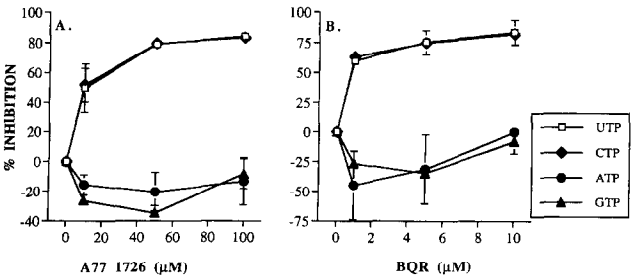

FIG. 6. Inhibition of pyrimidine nucleotide synthesis by A77 1726. LSTRA cells were grown in the absence or presence of various concentrations of A77 1726 (A) or BQR (B), and nucleotides were extracted and analyzed in an HPLC system as described in Materials and Methods. In the absence of inhibitors, the concentrations of UTP, CTP, GTP, and ATP were 872 ± 243, 125 ± 34, 1195 ± 335, and 2975 ± 1046 pmol/10^6 cells, respectively. The data are presented as mean percent inhibition from 3 independent experiments ± SEM.

Selective Inhibition of Pyrimidine Nucleotide Synthesis by A77 1726

The ability of A77 1726 to inhibit pyrimidine nucleotide synthesis was confirmed by quantitating nucleotide levels in LSTRA cells preincubated for 18 hr with various concentrations of A77 1726 or BQR. The results in Fig. 6A demonstrate that A77 1726 inhibited both UTP and CTP synthesis with an IC_{50} of 10 μM, whereas A77 1726 had no effect on ATP and slightly increased GTP levels. BQR also inhibited UTP and CTP pools with an IC_{50} of 0.8 μM, but enhanced the accumulation of both GTP and ATP pools (Fig. 6B). The difference in purine nucleotide levels was small in comparison with the associated error and was presumed not to be significant. Thus, compared with BQR, A77 1726 was about 10 times less potent at inhibiting pyrimidine nucleotide synthesis.

Direct Inhibition of DHO-DHase Activity by A77 1726

It has been suggested that A77 1726, similar to BQR, specifically inhibits the activity of DHO-DHase, the fourth enzyme in the *de novo* pyrimidine nucleotide pathway [4, 7]. *In vitro* DHO-DHase assays were performed in which the conversion of dihydroorotic acid to orotate was monitored directly. The results in Fig. 7 show that A77 1726 indeed inhibited DHO-DHase activity with an IC_{50} of 200 nM. Thus, A77 1726 was about 9-fold less potent than the positive control, BQR (IC_{50} = 25 nM), in inhibiting DHO-DHase activity.

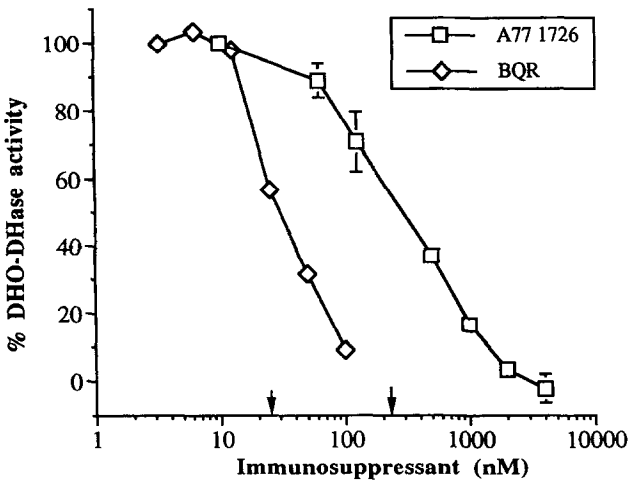

FIG. 7. Inhibition of DHO-DHase activity by A77 1726. Purified mitochondria from LSTRA cells were preincubated with various concentrations of A77 1726 or BQR for 30 min, and then the substrate, dihydroorotic acid, was added to initiate the enzymatic reaction as described in Materials and Methods. The percent DHO-DHase activity was calculated using the formulae:

$$\%\ \text{DHO-DHase activity} = 100 - \left[100 \times \frac{(\text{OD experimental sample} - \text{OD untreated sample})}{(\text{OD blank} - \text{OD untreated sample})}\right]$$

The OD_{600} absorbance value of the untreated control (in the absence of A77 1726 or BQR) was 0.529 ± 0.03, whereas the OD_{600} absorbance value of the blank control (in the absence of substrate, dihydroorotic acid) was 1.161 ± 0.05. The IC_{50} values of A77 1726 or BQR are indicated by the arrows. The data (mean ± SEM) from one of three representative experiments are presented.

DISCUSSION

The results in this paper demonstrate that A77 1726, an immunosuppressive agent, is able to inhibit the spontaneous growth and proliferation of the murine T cell line LSTRA. The IC_{50} values for inhibition of proliferation, measured by cell counts and [^{3}H]thymidine uptake after 24 hr of culture with A77 1726 (the approximate doubling time for LSTRA cells), were approximately 30 and 15 μM, respectively, while 90% inhibition of cell proliferation was observed at approximately 50 μM A77 1726.

It has been reported recently that the anti-proliferative activities of A77 1726 can be reversed by the addition of uridine [4–6]. In addition, it was reported that DHO-DHase was the high affinity binding protein for A77 1726, and it was proposed that inhibition of this enzyme was an *in vivo* mechanism of action of A77 1726 [7, 8]. We here confirm those *in vitro* observations and report that inhibition of LSTRA cell proliferation by A77 1726 can also be reversed by uridine. Analyses of intracellular nucleotide pools revealed that A77 1726 could selectively deplete UTP and CTP but not GTP and ATP in LSTRA cells. The *in vitro* DHO-DHase assay revealed that A77 1726 specifically inhibited DHO-DHase activity. The IC_{50} value of A77 1726 to inhibit DHO-DHase in our experiment was approximately 220 nM and was slightly higher than that reported by Williamson *et al.* [7] (IC_{50} = 81 ± 12 nM) using the same protocol except that the mitochondria were prepared from mouse spleen in their experiments. In contrast, Greene *et al.* [8] reported that A77 1726 inhibits DHO-DHase activity with an IC_{50} value of 2.7 ± 0.7 μM, which is about 10-fold higher than ours. The discrepancies in IC_{50} values could reflect the different methods used for the measurement of DHO-DHase activity. Nevertheless, these data collectively suggest that A77 1726 interferes with pyrimidine nucleotide synthesis via the inhibition of DHO-DHase activity, and despite the lack of structural similarity with BQR, these *in vitro* observations suggest that A77 1726 resembles BQR in its mode of action [16–19].

In the LSTRA cell line, $p56^{lck}$ protein kinase is overexpressed by approximately 40-fold compared with normal murine T lymphocytes [13]. It has been hypothesized previously that increased protein tyrosine phosphorylation contributes to the transformation of these cells [10]. We tested whether the ability of A77 1726 to inhibit LSTRA proliferation could be explained by inhibition of $p56^{lck}$ activity and reduction of intracellular tyrosine phosphorylation. Consistent with our previous report that A77 1726 has the ability to inhibit *src*-related PTK activity [3], A77 1726 was able to inhibit tyrosine phosphorylation in LSTRA cells and to inhibit immunoprecipitated $p56^{lck}$ activity quantitated by *in vitro* tyrosine kinase assays. The IC_{50} values for inhibition of total cellular tyrosine phosphorylation, after preincubating LSTRA cells for 6 hr with A77 1726, was approximately 50–100 μM. Thus, the concentrations of A77 1726 required for the inhibition of tyrosine phosphorylation were higher than those required for the inhibition of cell proliferation, and argued against the hypothesis that the mechanism by which A77 1726 inhibits LSTRA cell proliferation is by inhibition of PTK activity.

We also tested whether the ability of A77 1726 to inhibit tyrosine kinase activity was independent of inhibition of pyrimidine nucleotide synthesis. The ability of A77 1726 to reduce intracellular tyrosine phosphorylated proteins in LSTRA cells was not reversed by uridine. In addition, the ability of A77 1726 to inhibit the kinase activity of immunoprecipitated $p56^{lck}$, as assayed in an *in vitro* kinase assay, was also unaffected by the addition of uridine. Thus, A77 1726 has at least two separate effects on cells–inhibition of cellular tyrosine phosphorylation and inhibition of pyrimidine nucleotide synthesis. Demonstration that a pharmacological agent has multiple activities is not unexpected. Genistein was first identified as a specific inhibitor of PTK activity [20] but was later demonstrated to also inhibit mammalian DNA topoisomerase II activity [21]. More recently, Forrest *et al.* [22] reported that BQR completely inhibits IL-2 production and partially inhibits IL-2 receptor expression in T cells stimulated with phytohemagglutinin, and suggested that immunosuppression by BQR may be mediated by inhibition of IL-2 production as well as inhibition of pyrimidine nucleotide synthesis.

The observations that the IC_{50} of A77 1726 for inhibition of DHO-DHase was 220 nM and at least 200 times lower than that for inhibition of $p56^{lck}$ activity suggest that inhibition of LSTRA cell growth and proliferation by A77 1726 *in vitro* is predominantly mediated by the inhibition of pyrimidine nucleotide synthesis. However, the observations that the antiproliferative effects of A77 1726 on some cells, such as human T cells (PBLs) and the IL-2-dependent CTLL-4 cell line, can be reversed only partially by addition of uridine suggest that inhibition of tyrosine kinase activity may contribute to the inhibition of proliferation in these cells [23]. In addition, the ability of A77 1726 to inhibit the expression of cytotoxicity in $p56^{lck}$-transfected CTLL-2 cells is unaltered by the addition of uridine, and the IC_{50} for inhibition of cytotoxicity corresponds to that for inhibition of tyrosine kinase activity (Elder R and Chong A, manuscript in preparation), further suggesting a role for tyrosine kinase inhibition by A77 1726.

In conclusion, our findings confirm that A77 1726 has two separate activities: inhibition of the *de novo* pathway of pyrimidine nucleotide synthesis and interference with tyrosine phosphorylation. They further indicate that interference with pyrimidine nucleotide synthesis is the dominant mechanism by which A77 1726 inhibits LSTRA cell proliferation *in vitro*.

We are grateful to Dr. Tamara Hurley for providing us with the LSTRA cell line, to Dr. Robert Bartlett (Hoechst AG, Wiesbaden, Germany) for providing us with A77 1726 and BQR, and to Drs. Robert Elder and Ian Boussy for critical reading of the manuscript. This project was partially supported by a grant from the NIH (AI 34061).

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