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In Vitro and *In Vivo* Antitumor Activity of a Novel Immunomodulatory Drug, Leflunomide

MECHANISMS OF ACTION

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ABSTRACT. Leflunomide, a novel immunomodulatory drug, has two biochemical activities: inhibition of tyrosine phosphorylation and inhibition of pyrimidine nucleotide synthesis. In the present study, we first showed that A77 1726 [*N*-(4-trifluoromethylphenyl-2-cyano-3-hydroxycrotoamide)], the active metabolite of leflunomide, was more effective at inhibiting the tyrosine kinase activity of platelet-derived growth factor (PDGF) receptor than that of epidermal growth factor (EGF) receptor, and had no effect on the tyrosine kinase activity of the fibroblast growth factor receptor. In the presence of exogenous uridine, A77 1726 was more effective at inhibiting the PDGF-stimulated proliferation of PDGF receptor-overexpressing C6 glioma than the EGF-stimulated proliferation of EGF receptor-overexpressing A431 cells. *In vivo* studies demonstrated that leflunomide treatment strongly inhibited the growth of the C6 glioma but had only a modest effect on the growth of the A431 tumor. Uridine co-administered with leflunomide did not reverse the antitumor activity of leflunomide on C6 and A431 tumors significantly. Quantitation of nucleotide levels in the tumor tissue revealed that leflunomide treatment significantly reduced pyrimidine nucleotide levels in the fast-growing C6 glioma but had no effect on the relatively slow-growing A431 tumor. Whereas uridine co-administration normalized pyrimidine nucleotide levels, it had minimal effects on the antitumor activity of leflunomide in both tumor models. Immunohistochemical analysis revealed that leflunomide treatment significantly reduced the number of proliferating cell nuclear antigen-positive cells in C6 glioma, and that uridine only partially reversed this inhibition. These results collectively suggest that the *in vivo* antitumor effect of leflunomide is largely independent of its inhibitory effect on pyrimidine nucleotide synthesis. The possibility that leflunomide exerts its antitumor activity by inhibition of tyrosine phosphorylation or by a yet unidentified mode of action is discussed. BIOCHEM PHARMACOL **58**;9:1405–1413, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. leflunomide; protein tyrosine kinase; pyrimidine nucleotide synthesis; tumors

Growth factor receptors, such as EGF† receptor, TGF receptor α, and PDGF receptor, are expressed highly and frequently in epithelial tumors as well as in tumor cell lines [1–3]. In some tumors, the expression of these receptors is associated with a more aggressive clinical behavior and poor prognosis [4, 5]. These observations suggest that PTK inhibitors targeting growth factor receptors may be developed as novel therapeutic reagents for the control of the tyrosine kinase-dysregulated tumors. Indeed, several specific tyrosine kinase inhibitors have exhibited great efficacy in the control of tyrosine kinase-dysregulated tumors in preclinical studies. For example, tyrphostins have been found to be successful antiproliferative agents and to be largely nontoxic to cells grown in culture [6–8]. *In vivo* studies show that tyrphostins inhibit the growth of a human squamous cell carcinoma overexpressing the EGF receptor, when implanted into nude mice. Tyrphostins also are able to inhibit tumor growth and to prolong the survival of the mice [9]. A specific inhibitor of Abl tyrosine kinase is able to control the development of an *abl* gene-transformed tumor but has no effect on a *src* gene-transformed tumor [10]. Overall, these promising results have warranted further clinical trials of specific PTK inhibitors for the control of tyrosine kinase-dysregulated tumors.

Leflunomide (HWA 486 or SU101), a novel immunomodulatory drug, is currently being evaluated in phase I clinical trials for the control of kidney transplant rejection and phase II clinical trials for the treatment of patients with brain tumors [11, 12]. Leflunomide (Arava™) has been

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† *Abbreviations*: CMC, carboxylmethyl cellulose; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; HBSS, Hanks' balanced salt solution; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfphenyl)-2*H*-tetrazolium, inner salt; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PMS, phenazine-methosulfate; PTK, protein tyrosine kinase; and TGF, transforming growth factor.

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approved recently in Europe and in the U.S.A. for the treatment of patients with rheumatoid arthritis [13]. Extensive *in vitro* studies revealed that the active metabolite of leflunomide, A77 1726 [*N*-(4-trifluoromethylphenyl-2-cyano-3-hydroxycrotoamide)], has two biochemical activities: inhibition of tyrosine phosphorylation and inhibition of pyrimidine nucleotide synthesis [14–21]. The ability of A77 1726 to inhibit pyrimidine nucleotide synthesis is about 10–100 times more potent than its ability to inhibit tyrosine phosphorylation. In addition, uridine is able to reverse A77 1726-mediated inhibition of proliferation completely in some cell types [16, 17, 20]. These observations have led some investigators to propose that the *in vivo* mechanism of action of leflunomide is primarily through the inhibition of pyrimidine nucleotide synthesis [18, 22, 23]. However, our recent studies show that leflunomide treatment prevents lymphadenopathy and autoimmune disease in MRL/MpJ-*lpr*/*lpr* mice by a mechanism independent of the pyrimidine nucleotide synthesis pathway and that inhibition of tyrosine phosphorylation by A77 1726 may play a critical role in the antiproliferative and immunosuppressive activity of leflunomide *in vivo* [21]. Our present study employed a PDGF receptor-overexpressing C6 glioma and an EGF receptor-overexpressing A431 squamous carcinoma to investigate the *in vitro* and *in vivo* mechanisms of leflunomide-mediated antitumor activity.

MATERIALS AND METHODS

Reagents

The active metabolite of leflunomide, A77 1726 (SU020), was a gift from Dr. Robert Bartlett (Hoechst Marion Roussel). A77 1726 (10 mM) was dissolved in distilled water and stored at 4° for up to 2 weeks. Leflunomide was custom-synthesized and dissolved in a carrier solution of 1.5% CMC. Uridine was purchased from the Sigma Chemical Co. and dissolved in 0.9% NaCl. PDGF, EGF, bFGF, monoclonal anti-PDGF receptor antibody, and anti-phosphotyrosine mAb 4G10 were purchased from UBI. The anti-EGF receptor mAb was prepared from a hybridoma cell line, 579 (American Type Culture Collection), by protein G/A affinity chromatography (Oncogene Science). The anti-PCNA mAb was purchased from Oncogene Science. The biotin-conjugated goat anti-mouse IgG was purchased from The Jackson Immuno Research Laboratories, Inc. Horseradish-peroxidase streptavidin conjugate was purchased from ZYMED Laboratories Inc. Pansorbin was purchased from Calbiochem. ATP, CTP, UTP, and GTP were purchased from Sigma. A CellTiter 96 aqueous nonradioactive cell proliferation assay kit containing the substrates MTS and PMS was purchased from Promega.

Cells

Swiss 3T3 and A431 cell lines were purchased from the American Type Culture Collection. A431 cells, a human squamous carcinoma, and Swiss 3T3 fibroblast cells were grown in complete DMEM containing 10% FBS. C6 cells, a rat glioma cell line (a gift of Dr. L. K. Shawver at Sugen Pharmaceuticals), were grown in complete DMEM with 10% FBS.

In Vitro *Cell Proliferation*

C6 and A431 cells were seeded in 96-well flat-bottom plates. At about 20% confluence, cells were treated with various concentrations of A77 1726 in the absence or presence of 200 μM uridine, and then were incubated at 37° in a humidified CO_2 incubator for 48 hr. To remove from the culture medium the A77 1726 that may interfere with the substrate of the MTS assay, cells were washed twice with warm HBSS, and then were incubated with drug-free medium for 1 hr more, allowing intracellular A77 1726 to be released to the medium. Culture medium was then replaced by fresh medium containing the substrates MTS (20 μL/well in 200 μL of medium) and PMS (1 μL/well) (Promega). After incubation for 2–4 hr, the plates were read in an ELISA plate reader at OD_{490}. Wells without cells, filled with the culture medium containing substrates, were used as blanks.

Western Blot

Swiss 3T3 cells were seeded in 6-well plates (Costar) in DMEM containing 10% FBS. Upon 90% confluence, cells were washed twice with warm HBSS, and then starved overnight in DMEM containing 0.5% FBS. Next, cells were preincubated with the indicated concentrations of A77 1726 for 2 hr and stimulated with PDGF (20 ng/mL), EGF (20 ng/mL), or FGF (20 ng/mL) for 5 min. Cells were washed twice with cold PBS and then lysed in 200 μL of Brij 96 buffer (50 mM Tris–HCl, pH 8.0; 2 mM EDTA; 0.15 M NaCl; 1% Brij 96 (v/v); 200 μM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride; and 10 μg/mL of each aprotinin and leupeptin). Cell lysates were prepared and separated on an 8% SDS–polyacrylamide gel, and then were transferred onto a nitrocellulose membrane. Protein tyrosine phosphorylation was detected by an anti-phosphotyrosine mAb (4G10) and ECL (Amersham).

In Vitro *Tyrosine Kinase Assays*

Swiss 3T3 cells and A431 cells grown in T-75 flasks were stimulated for 5 min with PDGF (10 ng/mL) and EGF (20 ng/mL), respectively. Then cells were washed twice with cold PBS; cell lysates were prepared and precleared with Pansorbin. EGF receptor and PDGF receptor were immunoprecipitated with specific antibodies. Prior to the kinase reaction, PDGF receptor was activated by the addition of PDGF at a final concentration of 20 ng/mL. The immunoprecipitates were preincubated with various concentrations of A77 1726 at 4° for 10 min in a total volume of 50 μL of PTK buffer (50 mM HEPES, pH 7.4; 10 mM $MgCl_2$, and 10 mM $MnCl_2$). Ten microcuries of [γ-^{32}P]ATP was added to

initiate the tyrosine kinase reaction. The kinase reaction was performed at room temperature for 10 min, and 50 μL of 2x sample buffer (20% glycerol (v/v); 2% SDS; 25% 4x stacking gel buffer; 5% β-mercaptoethanol; 0.0025% bromophenol blue) was added to stop the reaction. The mixture was separated on an 8% SDS-polyacrylamide gel; protein phosphorylation was visualized by exposure of the dried gel to X-Omat film (Sigma).

HPLC Analyses of Intracellular Nucleotide Pool

C6 and A431 tumors (60 mg per sample) were homogenized briefly in 540 μL of 0.4 M trichloroacetic acid (TCA). Nucleotides were extracted by centrifugation (20 min × 15,000 rpm in a Eppendorf tabletop centrifuge) and neutralization of supernatants with 0.5 M tri-*o*-octylamine in Freon 113. Nucleotides were analyzed in a Waters HPLC system with a 616 pump, a 600S gradient controller, a 717 plus autosampler, and a 996 PDA detector. 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). The corresponding peaks of four nucleotides were integrated, and the concentrations were calculated based on a standard curve.

In Vivo *Inhibition of Tumor Growth by Leflunomide*

BALB/c *nu/nu* mice were purchased from the Jackson Laboratories. C6 or A431 cells (3×10^6 per mouse) were inoculated s.c. in the right dorsal region of 4- to 6-week-old female BALB/c *nu/nu* mice. After tumor inoculation, mice were divided randomly into 4 groups, 6–10 mice per group. Mice in the control group were injected i.p. with saline (0.1 mL/day) and gavaged with CMC carrier liquid (0.1 mL/day). Leflunomide (35 mg/kg) was administered by gavage, and uridine (500 mg/kg) was injected i.p. twice daily. Tumor volumes were determined by caliper measurement, twice a week, and calculated based on the formula: length · width^2 · π ÷ 6.

Immunohistochemical Staining

Tumor samples were removed quickly and embedded in TissueTEK OCT compound. The samples then were snap-frozen in liquid nitrogen and stored at −70° until used. Microsections of 6 μm were cut at −20° and mounted on polylysine hydrobromide-precoated slides. Slides were fixed in acetone at −20° for 10 min and air dried, then re-fixed in 1% paraformaldehyde and air dried for 2 min. To block endogenous peroxidase, slides were treated with 100% methanol for 5 min, and then with 0.15% H_2O_2 for 30 min at room temperature. Sections were incubated serially with the blocking serum, and then incubated for 1 hr at 37° with the anti-PCNA mAb. The sections were washed and incubated with biotinylated goat anti-mouse IgG for PCNA for 30 min, followed by horseradish peroxidase-conjugated streptavidin for 30 min. Chromogen, 3,3′-diaminobenzidine solution, was added, and the slides were counterstained with Mayer's hematoxylin. Finally, slides were washed, dehydrated, cleared, and mounted for microscopic examination.

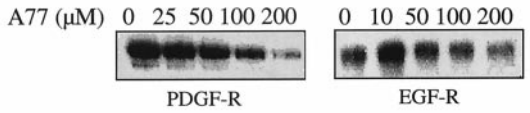

FIG. 1. Inhibition of autophosphorylation of PDGF receptor and EGF receptor by A77 1726. PDGF receptor and EGF receptor were immunoprecipitated with specific mAbs from Swiss 3T3 cells and A431 cells, respectively. PDGF receptor kinase in the immunoprecipitates was preactivated with PDGF. PDGF receptor and EGF receptor immunoprecipitates then were preincubated with the indicated concentrations of A77 1726, and protein tyrosine kinase assays were performed as described in Materials and Methods. The [^{32}P]-phosphorylated PDGF receptor (left panel) and EGF receptor (right panel) were detected by autoradiography.

Quantitation of Tyrosine Phosphorylation and Calculation of the IC_{50} Values

The exposed X-Omat films from the *in vitro* tyrosine kinase assays or the phosphotyrosine proteins detected on western blots were scanned in a Personal Computing Laser Densitometer (Molecular Dynamics). The peaks corresponding to the bands of interest were integrated to determine the relative amounts of phosphorylation. The IC_{50} values of A77 1726 required to inhibit the autophosphorylation of the PDGF receptor and the EGF receptor as well as of an approximately 130-kDa intracellular protein in PDGF- and EGF-stimulated fibroblast cells were determined from densitometric data of three independent experiments.

Statistical Analysis

All analyses to determine the significant differences between treated and control groups were performed using the repeated measures ANOVA (SuperANOVA, Abacus Concepts Inc.). Significant differences were concluded at $P \leq 0.05$ by ANOVA and a *post hoc* Tukey's compromise test.

RESULTS

Ability of A77 1726 to Inhibit the Autophosphorylation of Growth Factor Receptors and Protein Tyrosine Phosphorylation

Our previous studies have demonstrated that A77 1726 is capable of inhibiting Src-related tyrosine kinases, p56^{lck} and p59^{fyn} [19, 20]. Here, we show that A77 1726 was able to inhibit the autophosphorylation of the PDGF receptor and the EGF receptor with IC_{50} values of 75 and 150 μM, respectively (Fig. 1). The ability of A77 1726 to inhibit the growth factor receptor tyrosine kinases differentially was tested further in the Swiss 3T3 fibroblast cell line. The

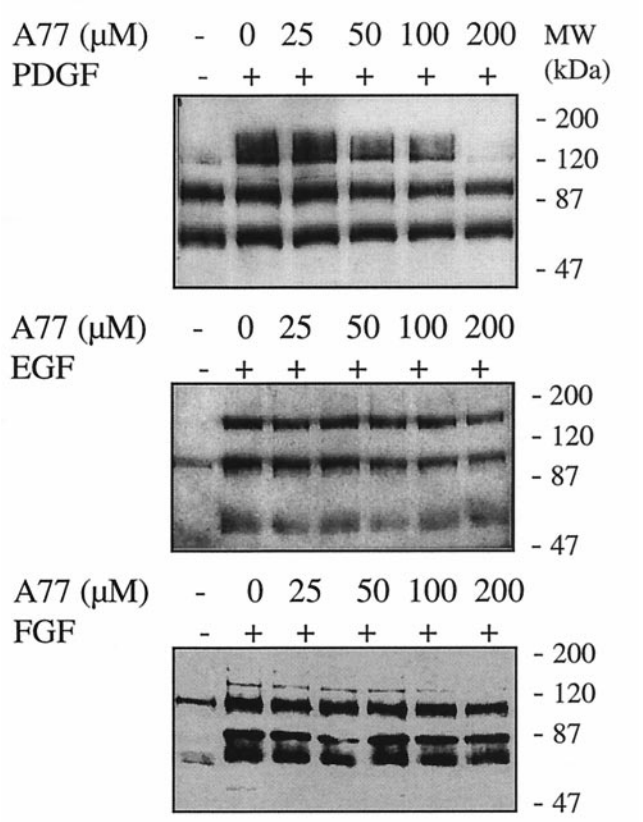

FIG. 2. Inhibitory effect of A77 1726 on protein tyrosine phosphorylation in Swiss 3T3 cells stimulated by PDGF, EGF, and FGF. Swiss 3T3 cells grown in 6-well plates were starved in DMEM containing 0.5% FBS overnight, then washed with HBSS twice and incubated in serum-free DMEM in the presence of the indicated concentrations of A77 1726 for 2 hr. The cells then were stimulated with 20 ng/mL of PDGF (top panel), EGF (middle panel), or FGF (bottom panel) for 5 min, cell lysates were prepared, and protein tyrosine phosphorylation was detected with an anti-phosphotyrosine mAb, 4G10, followed by ECL.

results in Fig. 2 show that A77 1726 inhibited PDGF-stimulated intracellular protein tyrosine phosphorylation in Swiss 3T3 cells with IC_{50} values ranging from 30 to 55 μM (top panel), whereas it inhibited EGF-stimulated intracellular protein tyrosine phosphorylation with IC_{50} values ranging from 150 to 200 μM (middle panel). Interestingly, A77 1726 was unable to inhibit FGF-stimulated protein tyrosine phosphorylation in Swiss 3T3 cells at concentrations up to 200 μM (Fig. 2, bottom panel). Consistent with previously published data [24], these results suggest that A77 1726 is able to inhibit the activity of growth factor receptor tyrosine kinases selectively.

In Vitro *Inhibition of Cell Proliferation by A77 1726*

Previous studies have demonstrated that A77 1726 is able to inhibit the cell proliferation of A431 cells with an IC_{50} value of 35 μM in a [^{3}H]thymidine uptake assay [25]. In the present study, we examined the ability of A77 1726 to inhibit the proliferation of PDGF receptor-overexpressing C6 glioma and EGF receptor-overexpressing A431 squamous carcinoma by using an MTS colorimetric assay. Our results in Fig. 3 show that A77 1726 inhibited the proliferation of PDGF-stimulated C6 glioma and EGF-stimulated A431 tumor with IC_{50} values of 0.1 and 10 μM, respectively. The addition of 200 μM uridine to the cell culture partially reversed the inhibition of proliferation of C6 and A431 cells and increased the IC_{50} values to approximately 20 and 100 μM, respectively. Thus, in the absence of exogenous uridine, A77 1726 inhibited the proliferation of C6 and A431 cells by inhibition of dihydroorotate dehydrogenase (DHO-DHase), whereas in the presence of exogenous uridine, A77 1726 may inhibit C6 and A431 cell proliferation by inhibition of the kinase activity of the PDGF receptor and the EGF receptor. At this time we cannot exclude the possibility that inhibition of cell proliferation in the presence of exogenous uridine may be due to an unidentified mechanism of action.

In Vivo *Inhibition of Tumor Growth by Leflunomide*

We then tested whether the ability of A77 1726 to inhibit tumor cell proliferation *in vitro* could be extrapolated to *in vivo* situations. The results in Fig. 4 show that treatment of nude mice with uridine (1 g/kg/day) alone slightly enhanced the growth of C6 glioma. Mice treated with leflunomide (35 mg/kg/day) inhibited C6 tumor growth by 91.7%, compared with that in the control group. Coadministration of uridine did not reverse leflunomide-

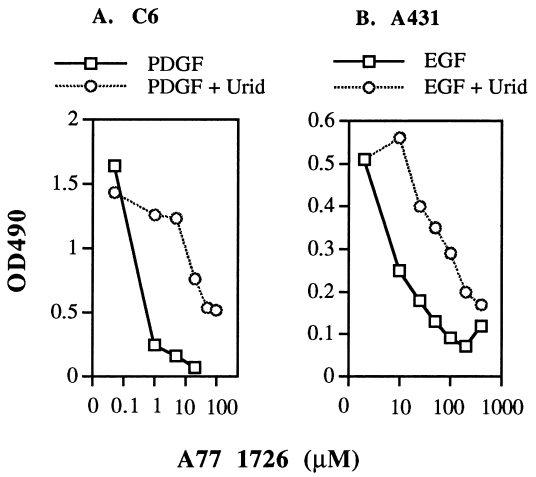

FIG. 3. In vitro inhibition of tumor cell growth by A77 1726. C6 and A431 cells were seeded in 96-well flat-bottom plates and grown in medium containing 0.5% FBS plus PDGF (20 ng/mL for C6 glioma) or EGF (20 ng/mL for A431 cells). Upon 20% confluence, A77 1726 was added at the indicated concentrations and incubated for 48 hr in the absence or presence of 200 μM uridine. Cells then were washed twice with HBSS and incubated with serum-free medium for 1 hr. Culture medium was removed and replaced by medium containing MTS and PMS substrates. The reaction was maintained at 37° for 1–4 hr, and then read at 490 nm in an ELISA reader. The data are the means of triplicate determinations from one of three separate experiments with similar results.

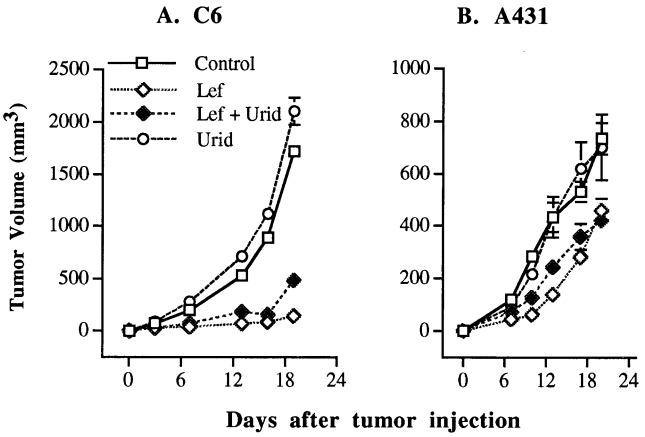

FIG. 4. In vivo inhibition of tumor growth by leflunomide. C6 and A431 cells (3×10^6 per mouse) were inoculated s.c. in the right dorsal region of 4- to 6-week-old female BALB/c *nu/nu* mice. Mice were divided randomly into 4 groups of 6–10 mice each. After 24 hr, mice were treated daily with the CMC carrier by gavage and with saline by i.p. injection as controls, or treated with leflunomide (35 mg/kg/day) by gavage and/or with uridine (1 g/kg/day) by i.p. injection. Tumor growth was monitored by caliper measurement, twice a week. Tumor volume was calculated based on the formula: length · width^2 · π ÷ 6. Results are the means ± SEM of tumor volumes from 4–6 mice. Abbreviations: Urid, uridine; and Lef, leflunomide.

mediated tumor growth inhibition during the first 2 weeks of treatment but slightly reversed leflunomide-mediated tumor growth inhibition by 21.6% at the end of treatment on day 19. Statistical analysis using repeated measures ANOVA and a *post hoc* Tukey's compromise test indicated that the reversal by uridine of the antitumor effect of leflunomide was not significant.

In contrast to the excellent antitumor activity of leflunomide on C6 glioma, leflunomide treatment (35 mg/kg/day) reduced the volumes of A431 tumors by only 37.5% after 20 days of treatment; statistical analysis using repeated measures ANOVA and a *post hoc* Tukey compromise test revealed that the inhibitory effect of leflunomide on the growth of the A431 tumor was significant. Co-administration of uridine did not affect the antitumor activity, suggesting that antitumor activity of leflunomide on the A431 tumor was not related to the inhibition of pyrimidine nucleotide synthesis and may be mediated by its inhibitory effect on EGF receptor kinase. These results further indicate that the efficacy of leflunomide-mediated tumor growth inhibition *in vivo* correlated with the inhibition of dysregulated protein tyrosine kinases expressed on tumor cells *in vitro*.

Effect of Leflunomide on the Pyrimidine Nucleotide Synthesis in Various Tumors

Our previous studies have demonstrated that leflunomide treatment does not reduce pyrimidine nucleotide levels in the lymphoid tissues of MRL/MpJ-*lpr/lpr* mice [21]. Here, we tested whether leflunomide and/or uridine reduced the nucleotide levels in the tumor tissue. The results in Table 1 show that treatment with uridine alone did not increase pyrimidine nucleotide levels significantly in C6 glioma or in A431 carcinoma ($P > 0.05$). Leflunomide treatment reduced the UTP and CTP levels in the rapidly proliferating C6 glioma ($P < 0.05$) but not in the relatively slowly proliferating A431 tumor ($P > 0.05$). Leflunomide and/or uridine treatment did not alter the ATP and GTP levels significantly in either C6 or A431 tumors, indicating that the reduction of pyrimidine nucleotides in C6 glioma in leflunomide-treated mice is specific. Co-administration of uridine with leflunomide normalized pyrimidine nucleotide levels in the C6 glioma but did not alter pyrimidine nucleotide levels in the A431 tumor significantly ($P > 0.05$). Since uridine in combination with leflunomide was able to normalize pyrimidine nucleotide synthesis but did

TABLE 1. Effect of leflunomide and uridine on intracellular nucleotide concentrations in C6 and A431 tumors grown in nude mice*

	Nucleotide levels (pmol/mg wet tumor)			
	UTP	CTP	ATP	GTP
C6 glioma				
Control	126 ± 20	167 ± 38	427 ± 59	58 ± 8
Uridine (1 g/kg/day)	172 ± 33	128 ± 22	526 ± 181	76 ± 13
Leflunomide (35 mg/kg/day)	40 ± 5†	28 ± 3†	339 ± 39	50 ± 4
Leflunomide (35 mg/kg/day) + uridine (1 g/kg/day)	133 ± 15	106 ± 11	560 ± 21	71 ± 3
A431 squamous carcinoma				
Control	82 ± 7	49 ± 4	298 ± 24	58 ± 4
Uridine (1 g/kg/day)	85 ± 7	49 ± 4	298 ± 24	58 ± 4
Leflunomide (35 mg/kg/day)	62 ± 4	33 ± 2	249 ± 14	47 ± 2
Leflunomide (35 mg/kg/day) + uridine (1 g/kg/day)	74 ± 4	45 ± 3	244 ± 10	49 ± 2

*C6 and A431 cells (3×10^6 per mouse) were inoculated s.c. in the right dorsal region of 4 to 6-week-old female BALB/c *nu/nu* mice. Mice were divided randomly into 4 groups, 4–6 mice each. After 24 hr, mice were treated daily with the CMC carrier by gavage and saline by i.p. injection as controls, or treated with leflunomide (35 mg/kg/day) by gavage and/or uridine (1 g/kg/day) by i.p. injection. Betweeen 2 and 4 hr after the last treatment, mice were killed, and nucleotides were extracted from tumor tissues and then quantitated in an HPLC system as described in Materials and Methods. The results represent means ± SEM from 4–6 mice in each group. This experiment was repeated twice, and similar results were obtained.

†$P \leq 0.05$, compared with that in the control group.

not reduce the antitumor activity of leflunomide significantly, we conclude that leflunomide-mediated antitumor activity is largely independent of its ability to inhibit pyrimidine nucleotide synthesis and may be due to its ability to inhibit PTK activity.

In Vivo *Inhibition of Cell Proliferation*

The cell proliferation of C6 tumors grown in nude mice treated with various reagents was examined by immunohistochemical staining with a specific antibody against PCNA. The results in Fig. 5 show that there was a mean of 58.3 ± 6.0 PCNA-positive cells per field in the C6 glioma from untreated control mice. Uridine by itself marginally increased the number of PCNA-positive cells, by 10.5%. Leflunomide treatment significantly reduced the number of PCNA-positive cells to a mean of 36.3 ± 6.7 PCNA-positive cells per field; co-administration of uridine increased the number of PCNA-positive cells to 46.6 ± 6.6, a 26.9% increase compared with that in the leflunomide-treated group. These observations indicate that uridine only partially reverses the antiproliferative effect of leflunomide on C6 glioma, and that a significant portion of leflunomide-mediated antitumor activity is not dependent on the reduction of pyrimidine nucleotide levels.

DISCUSSION

Previous studies by Mattar *et al.* [25] demonstrated that A77 1726 is able to inhibit the activity of EGF receptor kinase with IC_{50} values of 30–50 μM. Shawver *et al.* [24] recently reported that A77 1726 was able to inhibit PDGF receptor and vascular endothelial growth factor receptor kinases with IC_{50} values of 60 and 54 μM, respectively. However, A77 1726 was unable to inhibit EGF receptor kinase activity ($IC_{50} > 1000$ μM) in their ELISA-based tyrosine kinase assay system. Consistent with these observations, our *in vitro* tyrosine kinase assays showed that A77 1726 inhibited the autophosphorylation of PDGF with IC_{50} values of 50 μM but only weakly inhibited EGF receptor kinase activity ($IC_{50} = 150$ μM). We further demonstrated that A77 1726 was more potent at inhibiting the tyrosine phosphorylation in PDGF-stimulated Swiss 3T3 cells than in EGF-stimulated cells and had no effect on the FGF-stimulated tyrosine phosphorylation. These results collectively suggest that A77 1726 is a modest tyrosine kinase inhibitor with modest selectivity.

We and others have demonstrated that leflunomide has a strong second activity: inhibition of pyrimidine nucleotide synthesis. A77 1726 inhibits DHO-DHase activity about 10- to 100-fold more effectively than it inhibits tyrosine kinase activity [15–18, 20]. This raised the question of which activity of leflunomide is responsible for its antitumor activity. To address this, we first tested whether leflunomide had any anti-pyrimidine nucleotide effect *in vivo*. Our results in Table 1 show that leflunomide treatment indeed reduced pyrimidine nucleotide levels in the C6 tumor tissue but did not result in reduced pyrimidine nucleotide levels in A431 tumor tissues. This discrepancy may be due to the difference in the tumor growth rate. The doubling time of C6 tumor growth was one-third of that for A431 carcinoma (compare panels A and B of Fig. 4). Therefore, in the presence of leflunomide, the salvage of pyrimidine nucleotide could be a limiting factor for the rapidly growing C6 glioma but not for the relatively slow-growing A431 carcinoma. In support of this notion, uridine treatment alone slightly stimulated the growth of C6 glioma but had no effect on the A431 tumor (Fig. 4). This hypothesis is consistent with our previous observations showing that treatment of lymphoproliferative disease in MRL/MpJ-*lpr/lpr* mice with leflunomide did not reduce pyrimidine nucleotide levels in the relatively slow-growing lymph nodes, compared with the growth rate of C6 glioma [21]. An alternative explanation for the differential effect of leflunomide on the pyrimidine nucleotide levels in C6 glioma and A431 tumor is that DHO-DHase of rat origin in C6 glioma is much more sensitive to A77 1726 than DHO-DHase of human origin in A431 tumor [15, 26]. Therefore, the *de novo* pyrimidine nucleotide synthesis in leflunomide-treated C6 glioma may be inhibited completely, whereas the *de novo* pyrimidine nucleotide synthesis in A431 tumor may be inhibited only partially.

Since leflunomide reduced intracellular pyrimidine nucleotide levels in C6 glioma *in vivo*, we reasoned that if the antitumor activity of leflunomide is mediated by inhibition of pyrimidine nucleotide synthesis, then co-administration of uridine that normalizes pyrimidine nucleotide levels in tumor tissues should antagonize the antitumor activity of leflunomide. Our results in Table 1 show that uridine co-administered with leflunomide indeed normalized pyrimidine nucleotide levels in tumor tissue. In contrast, the data in Fig. 4 demonstrate that uridine co-administered with leflunomide did not antagonize the leflunomide-mediated antitumor activity significantly ($P > 0.05$, statistically analyzed by repeated measures ANOVA). In addition, uridine co-administered with leflunomide did not alter the pyrimidine nucleotide levels in A431 tumor tissues or affect the antitumor activity of leflunomide. Based on these results, we concluded that the antitumor activity of leflunomide was largely unrelated to its effect on pyrimidine nucleotide synthesis and was mediated predominantly by its ability to inhibit tyrosine phosphorylation. This conclusion is consistent with our recent studies showing that the control of lymphoproliferative and autoimmune disease in MRL/MpJ-*lpr/lpr* mice by leflunomide is independent of its inhibitory effect on pyrimidine nucleotide synthesis and is correlated with the reduced $p59^{fyn}$-mediated protein tyrosine phosphorylation in the lymphoid tissues [21].

Tyrosine kinase inhibitors have received extensive attention in the past few years for their great potential to be developed as novel therapeutic agents for the control of a variety of PTK-dysregulated tumors [11]. Based on the ability of leflunomide to inhibit tyrosine kinase activity, leflunomide is currently being developed as a novel anti-

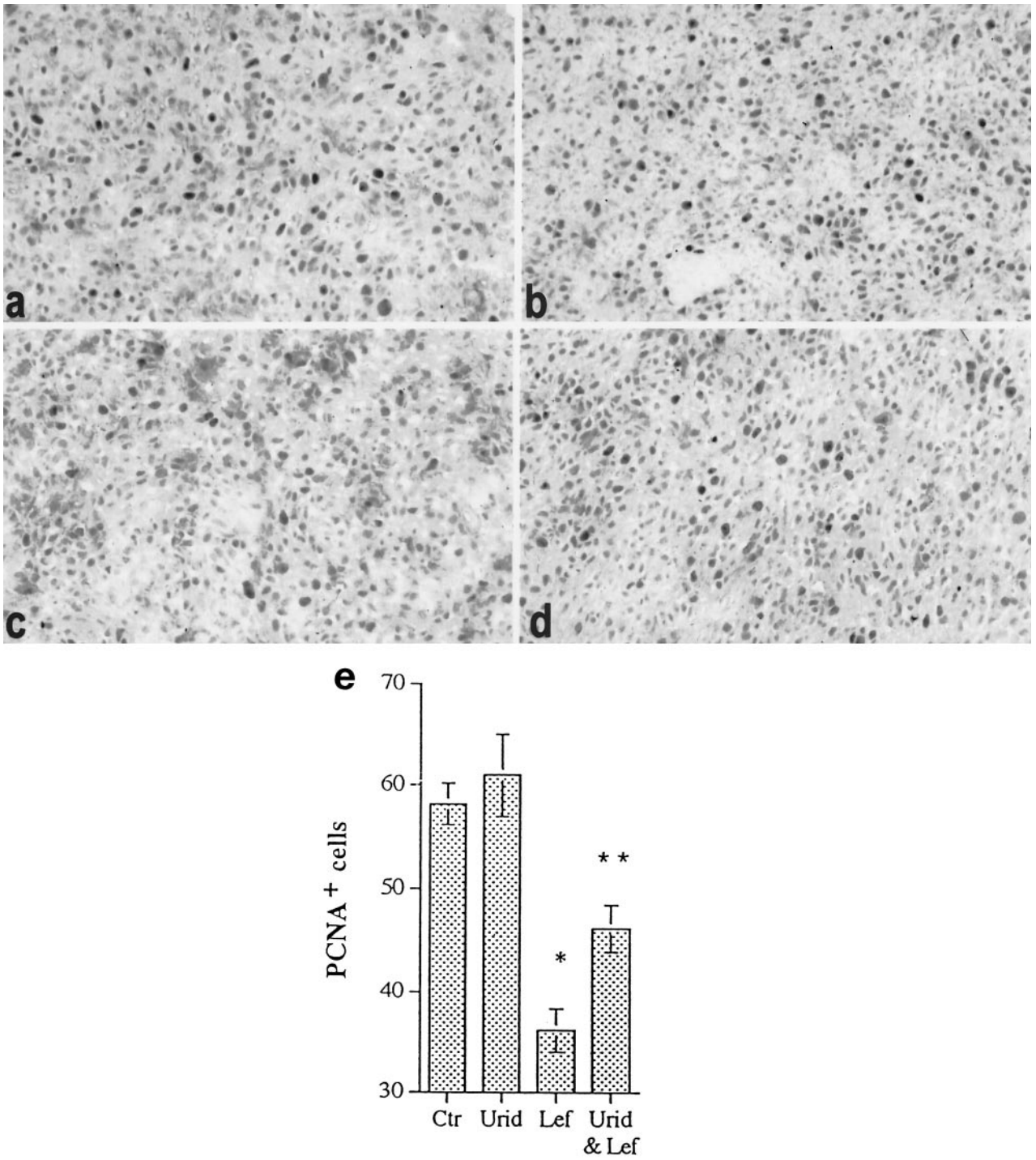

FIG. 5. In vivo inhibition of C6 glioma cell proliferation. C6 gliomas removed from control (a), uridine-treated (b), leflunomide-treated (c), and leflunomide plus uridine-treated (d) mice were sectioned and stained with anti-PCNA mAb as described in Materials and Methods. The proliferating cells were positively stained with mAb against PCNA. (e) Statistical analysis of PCNA-positive cells. PCNA-positive cells were counted from 10–20 fields (400x magnification) of each tumor sample. Results are the means ± SEM of PCNA-positive C6 glioma cells per microscope field from four animals per group. Key: (*) $P < 0.05$ compared with the untreated control; and (**) $P < 0.05$ compared with the leflunomide-treated group.

cancer drug. Leflunomide is the first PTK inhibitor currently tested in clinical trials for the treatment of patients with brain tumor and other PTK-dysregulated tumors [11, 12]. Our present studies investigated the mechanism of the *in vivo* antitumor activity of leflunomide on two different tumors, C6 glioma, which expresses high levels of PDGF receptor, and an A431 tumor, which expresses high levels of EGF receptor. Our results show that leflunomide was more effective at inhibiting the growth of the C6 tumor than that of the A431 tumor (Fig. 4). In addition, lefluno-

mide had no inhibitory effect on the growth of HT29 cells, a human colon adenocarcinoma in which no PTKs reportedly are dysregulated (Xu X, unpublished observations). These data are consistent with previous observations by Shawver *et al.* [24] showing that leflunomide is highly effective at inhibiting the growth of several PDGF receptor-expressing tumors but only weakly inhibits the growth of EGF receptor-expressing tumors, including the A431 tumor. These results collectively suggest that the leflunomide-mediated antitumor activity correlates with its *in vitro* ability to inhibit the dysregulated tyrosine kinases expressed in tumor cells, and suggests that the *in vivo* antitumor activity of leflunomide may be related, at least in part, to the regulation of protein tyrosine phosphorylation. This hypothesis is supported by our earlier studies [21] showing that leflunomide inhibits the activity of $p59^{fyn}$ tyrosine kinase with an IC_{50} of approximately 50 μM in lymphocytes of MRL/MpJ-*lpr/lpr* mice and prevents the lymphoproliferative and autoimmune disease at a dose of 35 mg/kg/day.

The IC_{50} of leflunomide required to inhibit PDGF receptor tyrosine kinase activity is relatively high (50–60 μM); however, leflunomide possesses an excellent *in vivo* antitumor activity. In contrast, several small molecules with the ability to specifically inhibit PTKs at IC_{50} values in the nanomolar range, such as the Abl-specific inhibitor CGP 57148 and the PDGF receptor-specific inhibitor CGP 53716 [10], require dosages comparable to that of leflunomide to achieve good *in vivo* antitumor activity. This raises the possibility that the *in vivo* mechanism of action of leflunomide may not be due to the inhibition of PDGF receptor kinase activity *per se* but to the inhibition of some other PTKs or non-PTK targets. A second possibility is that the pharmacokinetics of leflunomide is different from that of the other potent PTK inhibitors. The serum concentration of A77 1726 in mice treated with leflunomide at a dose of 35 mg/kg had a remarkably long half-life of 15 hr (Chong AS-F, unpublished data). A77 1726 peaks at 500 μM within 4 hr and remains at 250 μM at 24 hr after a single dose of 35 mg/kg of leflunomide. Both these concentrations are theoretically sufficient to inhibit PDGF receptor tyrosine kinase, as analyzed by *in vitro* tyrosine kinase assays and in cell culture.

In summary, our results demonstrated that *in vitro* antitumor activity of A77 1726 in the absence of exogenous uridine was mediated largely by the inhibition of pyrimidine nucleotide synthesis, whereas in the presence of exogenous uridine, inhibition of cell proliferation was mediated mainly by the inhibition of tyrosine phosphorylation. However, the possibility that an unidentified mechanism of action of leflunomide may be responsible for its anti-proliferative activity cannot be excluded at this time. The *in vivo* antitumor activity of leflunomide correlated with its inhibitory effect on the dysregulated tyrosine kinases expressed by tumors. In addition, our results show that uridine had little effectiveness at antagonizing the antitumor activity of leflunomide. These observations suggest that the control of tyrosine kinase-dysregulated tumors by leflunomide is independent of the inhibition of pyrimidine nucleotide synthesis and may be mediated by inhibition of tyrosine phosphorylation or by an as yet unidentified mode of action.

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