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Inhibition of the epidermal growth factor receptor tyrosine kinase activity by leflunomide

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The active metabolite of leflunomide, A77 1726 inhibits the proliferation of a variety of mammalian cell lines in culture. Epidermal growth factor (EGF)-dependent proliferation is inhibited by A77 1726 at an effective dose of 30–40 μM. A77 1726 appears to directly inhibit the EGF receptor tyrosine-specific kinase activity both in intact cells and purified EGF receptors at the same effective dose. These data suggest that leflunomide inhibits cellular proliferation by the inhibition of tyrosine-specific kinase activities.

Epidermal growth factor receptor; Tyrosine-specific kinase; Leflunomide; Human foreskin fibroblast cell

1. INTRODUCTION

A variety of cell surface receptors, such as the epidermal growth factor (EGF) receptor, posses a ligand-sensitive intrinsic tyrosine-specific kinase activity [1,2]. Ligand binding to one of these receptors leads to tyrosine phosphorylation of intracellular substrates and are thought to be one of the intracellular signals necessary for DNA synthesis and cell division. Several laboratories are investigating tyrosine-specific protein kinase inhibitors as an approach to cell growth inhibition. A variety of compounds from natural origins, such as flavonoids [3] or erbstatin [4], as well as synthetic compounds [5,6], have been reported to inhibit tyrosine kinase activity. Thus, specific inhibitors of tyrosine kinases are of great interest as therapeutic agents and pharmacologic tools to study tyrosine phosphorylation and its role in cell growth control.

Leflunomide, a novel immunomodulatory drug, has been shown to be effective in the treatment of several autoimmune animal diseases with minimal toxicity [7,8]. The in vitro investigations concerning its mode of action demonstrated that leflunomide mediates its effects, at least in part, by inhibition of cellular proliferation of a wide variety of cultured cell lines [8]. These data suggested that leflunomide might interfere with a common mechanism leading to cell division. The importance of tyrosine kinases in the regulation of cell proliferation and the structure of leflunomide led us to examine its ability to inhibit tyrosine-specific protein kinase activity of growth factor receptors.

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2. MATERIALS AND METHODS

The active metabolite of leflunomide, A77 1726, was supplied by Hoechst, AG, Weisbaden, Germany. Prior to use it was dissolved directly into the buffer and used without further purification.

2.1. *Cell culture*

The human foreskin fibroblast (HFF) and squamous cell carcinoma (KB or A431) cell lines used for these experiments were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% iron-supplemented calf serum. Murine splenic T- and B-cells, a cytotoxic T-cell line (CTLL), and B-cell lines (A20–2-J or 7D4) were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum. All cultures were maintained at 37°C in an humidified atmosphere with 5% CO_2. Cellular proliferation was measured by the incorporation of thymidine into DNA after a 24 h pulse with [^{3}H]TdR (1 μCi/well; ICN Radiochemicals) [9]. The results are expressed as the mean of four replicate cultures. The maximum standard deviation (S.D.) for each point was ≤ 15%.

2.2. *Phosphorylation of the EGF receptor in intact cells*

Phosphorylation of the EGF receptor in intact cells was determined with an anti-phosphotyrosine antibody as previously described [10] and outlined below. Human foreskin fibroblast cells (3×10^5) were seeded in 25 cm^2 flasks in serum-free medium supplemented with 20 ng/ml EGF (UBI) and with or without A77 1726. The cells were cultured for 3 days and then transferred to serum-free medium supplemented with A77 1726 alone for an additional 24 h. The EGF receptor was stimulated with 100 ng EGF per flask for 10 min at 37°C. After washing 2× with ice-cold phosphate buffered saline (PBS), the monolayer was solubilized with buffer containing 1% Triton-X-100, 10% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.4, 1 mM EGTA, 10 mM iodoacetic acid and 100 μM phenylmethylsulfonyl fluoride (PMSF), for 10 min.

After determination of the protein concentration for the lysates, 50 μg of loaded soluble protein was resolved by SDS-PAGE and then

transferred electrophoretically to nitrocellulose sheets. Phosphotyrosine-containing proteins were identified with an anti-phosphotyrosine monoclonal antibody (PY-20, ICN Immunochemicals) and detected by sequential blotting with biotinylated goat anti-mouse IgG, streptavidin alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyphosphate *p*-toluidine salt/nitroble tetrazolium chloride (BCIP/NBT) (Bethesda Research Labs).

2.3. *Determination of the EGF receptor kinase activity*

The tyrosine-specific protein kinase activity of the EGF receptor was determined as described previously using a synthetic peptide substrate [11]. For these determinations, the EGF receptor was purified from A431 cells by affinity chromatography on immobilized wheat germ agglutanin as previously described [12]. The EGF receptor was specifically eluted with 3 mM *N*-acetyl glucosamine and dialized prior to use. The kinase reaction was carried out in a total volume of 50 μl in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$ and 100 μM sodium orthovanadate. Purified EGF receptor (15 μl) was preincubated with or without 50 ng EGF and A77 1726 for 20 min to initiate the kinase assay. The kinase reaction was started by the addition of [γ^{32}P]ATP at 4°C for 5 min to allow for autophosphorylation. The synthetic peptide substrate (RR-src) (Peninsula Labs) was then added and the reaction allowed to proceed for an additional 10 min at 4°C. The reaction was stopped by the addition of 50 μl ice-cold 10% trichloroacetic acid. Following centrifugation, the supernatant containing the phosphorylated RR-src peptide was transferred onto phosphocellulose paper (Whatman P81) and washed three times in 75 mM phosphoric acid. The dried papers were then counted with a liquid scintilation counter. The results are expressed as the mean of three seperate experiments for each data point.

2.4. *Determination of protein kinase C activity*

Protein kinase C activity was measured using the PKC Assay System purchased from Gibco BRL, and all proceedures were performed as per the manufacture's instructions. This assay system is based on the proceedure previously described by Yasuda et al. [13]. The PKC used for these determinations was purified from HL-60 cells by DEAE chromatography. Kinase activity was determined by incorporation of ^{32}P from [γ^{32}P]ATP (3,000 μCi/pmol) into a synthetic peptide substrate derived from myelin basic protein (provided with the assay kit). The specificity of the assay for PKC was confirmed by using the PKC pseudosubstrate inhibitor peptide, PKC (19–36) [13].

3. RESULTS

3.1. *Inhibition of EGF-dependent proliferation by A77 1726*

It was previously demonstrated that A77 1726 (Fig. 1) inhibits the proliferation of a wide variety of cell lines [8]. The ability of A77 1726 to inhibit the serum-dependent proliferation of leukocytes and tumor cell lines is summarized in Table I. All of these cell lines require tyrosine-specific kinase activity for proliferation. Inhibition of tyrosine-specific kinase activity could be a mechanism by which A77 1726 could inhibit cell growth. To investigate this possibility, an EGF-dependent human foreskin fibroblast cell line (HFF) was evaluated for the ability of A77 1726 to inhibit EGF-stimulated proliferation. HFF cells were incubated in the presence or absence of different concentrations of A77 1726 and after 4 days proliferation was measured by [^{3}H]thymidine incorporation. The concentration of A77 1726 required to inhibit 50% proliferation of HFF cells was approximately 30 μM (Fig. 2). Cell viability, as determined by Trypan blue exlusion, was unaffected at any of the drug concentrations used. The effect of A77 1726 on HFF cell growth was also reversable: 48 h after removal of A77 1726, EGF stimulated HFF cell growth and proliferation similar to control cultures (data not shown).

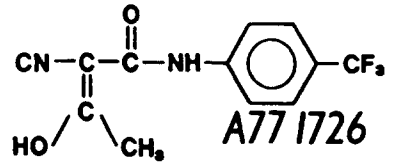

Fig. 1. The chemical structure of A77 1726.

3.2. *Inhibition of EGF receptor autophosphorylation by A77 1726*

Since A77 1726 appeared to inhibit EGF dependent cell growth, the effect of A77 1726 on the EGF receptor tyrosine-specific protein kinase activity was investigated. The results described in Fig. 3 indicate that A77 1726 can inhibit the autophosphorylation of the EGF receptor in intact cells. Stimulation of HFF cells for 10 min with EGF results in phosphorylation of a 170 kDa protein consistant with autophosphorylation of the EGF receptor (Fig. 3, Lanes 3 and 4). The identity of this band as the EGF receptor was confirmed by anti-EGF receptor monoclonal antibody (clone F4, Sigma) staining (data not shown). Human foreskin fibroblast cells incubated in the presence of 30 μM A77 1726 and stimulated with EGF demonstrated reduced auto-phosphorylation of the EGF receptor (Fig. 3, lanes 7 and 8). Cells grown in different concentrations of A77 1726 revealed that the effective dose for inhibition of autophosphorylation (20–30 μM, Fig. 4) was similar to the dose required for inhibition of cell growth (30 μM, Fig. 2).

3.3. *Inhibition of tyrosine kinase activity by A77 1726*

The ability of A77 1726 to directly inhibit the EGF receptor tyrosine-specific kinase was measured on purified EGF receptors. Fig. 5 shows the inhibition of tyrosine-specific kinase activity observed with increasing

Table I

Inhibition of cell proliferation by A77 1726

Cell line	Origin	Species	ED_{50} (μM)
CTLL	Cytotoxic T-cell (IL-2 dep.)	Murine	40[a]
A20-2-J	BALB/c B-cell tumor	Murine	3
T-cells and B-cells	Spleen	Murine	10
KB	Epidermoid tumor	Human	15
A431	Epidermoid tumor	Human	35
7D4	B-cell hybridoma	Rat	1

[a] Determined by [^{3}H]thymidine incorporation.

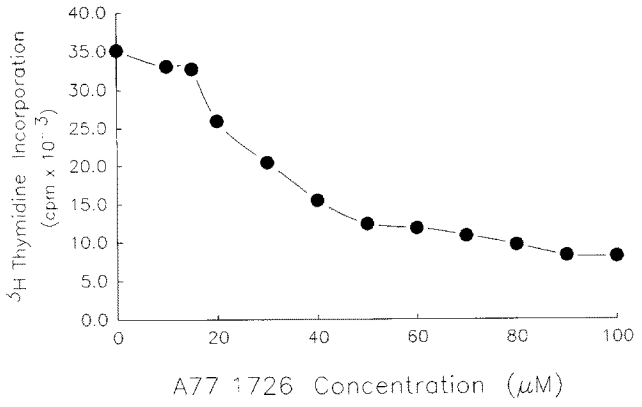

Fig. 2. Inhibition of [^{3}H]thymidine incorporation into human foreskin fibroblast cells by A77 1726.

concentrations of A77 1726. The inhibition curve was very similar to that seen for autophosphorylation in intact cells and inhibition of cell growth (IC_{50} 30–40 μM). These results demonstrated that A77 1726 inhibited the tyrosine-specific protein kinase activity of the EGF receptor. Moreover, these results suggested that the ability of A77 1726 to inhibit EGF-dependent cell growth may be related directly to its ability to inhibit the tyrosine kinase activity of the EGF receptor.

3.4. *Specificity of A77 1726 inhibition*

The data described above indicated that A77 1726 could inhibit the EGF receptor tyrosine kinase activity. The growth inhibition of the IL-2-dependent cytotoxic T-cell line (CTLL) suggested that A77 1726 may also be able to inhibit the kinase activity of the lyphocyte-specific src family kinase ($p56^{lck}$) (Table I) [15]. Additional data from the laboratory has indicated that A77 1726 can reduce IL-2-dependent tyrosine phosphorylation in these cells (D. Nikcevich and E. Bremer, unpublished observation). Platelet-derived growth factor-dependent tyrosine phosphorylation was also inhibited by A77 1726 in intact cells at concentrations similar to EGF-dependent phosphorylation described in Fig. 3 (data not shown).

These data suggested that A77 1726 can inhibit a variety of tyrosine-specific kinases. We also tested the ability of A77 1726 to inhibit a serine/theonine-specific kinase. Protein kinase C (PKC) was chosen as a model

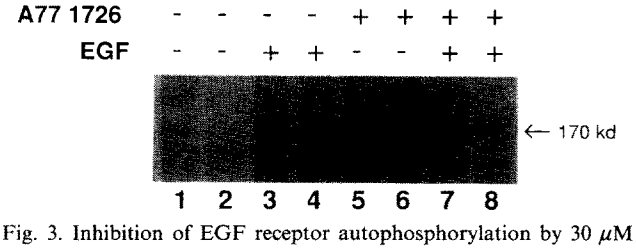

Fig. 3. Inhibition of EGF receptor autophosphorylation by 30 μM A77 1726.

Table II

Inhibition of protein kinase C activity by A77 1726

[A77 1726] (μM)	PKC activity[a] (pmol/min/10^6 cells)
0	14.2
25	13.4
50	14.5
100	15.0
150	13.8

[a] PKC activity was determined with PKC purified from the human myologenous leukemia cell line, HL-60. Enzyme activity was measured by the transfer of ^{32}P to a synthetic peptide substrate from ATP using the Gibco-BRL PKC assay kit as described in section 2.

serine/theonine-specific kinase since it can phosphorylate the EGF receptor and reduce the receptor's kinase activity [16]. A77 1726 was unable to inhibit PKC activity at concentrations ranging between 25 and 150 μM (Table II). At these concentrations, A77 1726 is able to strongly inhibit the EGF receptor kinase in both intact cells (Fig. 3) and in vitro (Fig. 5). These data indicate that A77 1726 is acting by directly inhibiting the tyrosine-specific kinase activity.

4. DISCUSSION

The data presented here demonstrates that A77 1726, the active metabolite of leflunomide, is able to inhibit EGF-dependent cell growth. This drug was also able to inhibit EGF receptor autophosphorylation in intact HFF cells. The concentration of drug required to inhibit autophosphorylation (20–30 μM) was approximately the same concentration required to inhibit HFF proliferation (30–40 μM). The effect of A77 1726 on the EGF receptor autophosphorylation was due to a decrease in kinase activity (Fig. 5). The concentration (approx. 40 μM) required for inhibition of the EGF receptor tyrosine-specific kinase activity was the same as that required for inhibition of cell growth, suggesting that inhi-

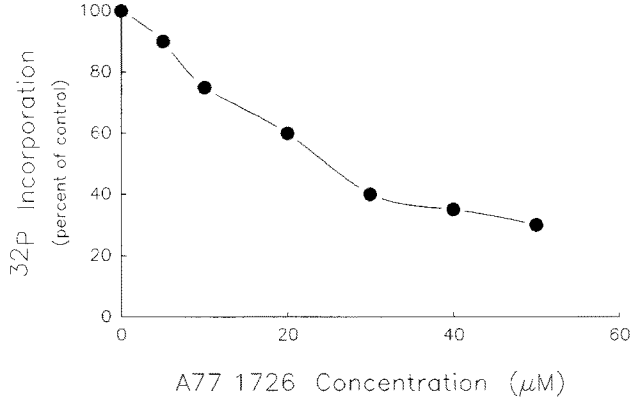

Fig. 4. Percent inhibition of EGF receptor autophosphorylation by various concentrations of A77 1726.

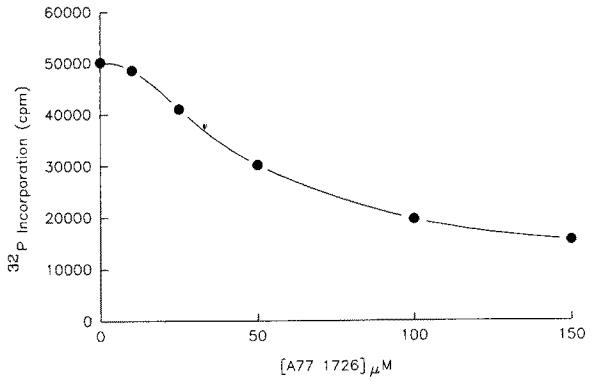

Fig. 5. Inhibition of the purified EGF receptor tyrosine-specific kinase activity by A77 1726.

bition of kinase activity may be the mechanism for growth inhibition by A77 1726.

A variety of compounds have been described as inhibitors of the EGF receptor tyrosine-specific kinase activity. In cell-free kinase assays, many of these compounds appear to be better inhibitors than A77 1726. Erbstatin has a K_i of 6.0 μM [12], genistein an IC_{50} of 2.6 μM for autophosphorylation of the EGF receptor [3], Tyrophostin RG50864 a K_i of 0.85 μM [5] and thiazoldine-diones an IC_{50} of 1–6 μM [13]. On the other hand, some of these kinase inhibitors are not nearly as effective in intact cells. Erbstatin and its analogs require greater than 130 μM concentrations to inhibit EGF receptor autophosphorylation in cultured A431 cells [4]. In this report, we describe cell growth inhibition by A77 1726 at 35 μM for A431 cells and 30 μM for HFF cells. Furthermore, EGF receptor autophosphorylation is inhibited by A77 1726 at 30 μM in intact cells. These data suggest that A77 1726 is a very effective inhibitor of the EGF receptor kinase in live cells.

Leflunomide was developed for its potential as an immunomodulatory drug and has been extensively studied in animal models [7,8]. The animal studies have indicated that leflunomide can inhibit graft rejection and adjuvant arthritis [7,8]. The ability of A77 1726 to inhibit the EGF receptor kinase suggests that the immunomodulation seen with this drug may be due to inhibition of tyrosine-specific protein kinase activities. Tyrosine kinase activity has been implicated in the signal transduction mechanisms of the T-cell receptor [17] and several cytokine receptors, including the IL-2 receptor [18]. In addition to leflunomide's potential as an immunomodulatory drug, the data presented there suggest that A77 1726 may be a useful tool for defining the role of tyrosine-specific kinases in the activation of T and B cells.

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