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Differential Modulation of Pro- and Anti-inflammatory Cytokine Receptors by N-(4-Trifluoromethylphenyl)-2-Cyano-3-Hydroxy-Crotonic Acid Amide (A77 1726), the Physiologically Active Metabolite of the Novel Immunomodulator Leflunomide

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ABSTRACT. N-(trifluoromethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide (A77 1726), the physiologically active metabolite of leflunomide, has been described to exert antiproliferative effects *in vitro* and anti-inflammatory actions in several animal models. Currently, its use is being evaluated in clinical trials in psoriasis, which is characterized by epidermal hyperproliferation and infiltration of inflammatory cells. We studied the effects of A77 1726 on growth and gene expression in cultured epidermal cells by 5-bromo-2′-deoxy-uridine (BrdU) incorporation, reverse transcriptase-polymerase chain reaction (RT-PCR), Northern blot hybridizations and flow cytometry. A77 1726 inhibited epidermal proliferation at concentrations above 5 μM after 24 hr. However, the cells were still fully viable at a concentration of 100 μM. The drug caused a dose-dependent reduction in the mRNA level of the type A receptor for the proinflammatory cytokine interleukin-8 (IL-8-RA) and, in contrast, induced gene expression of the receptor for the anti-inflammatory cytokine IL-10 (IL-10R) at the mRNA and protein levels. In addition, the mRNA and protein levels of the p53 gene, which is a negative cell cycle regulator, were up-regulated by A77 1726. These data suggest that A77 1726 exerts its anti-inflammatory action via the modulation of epidermal gene expression. BIOCHEM PHARMACOL **55**;9:1523–1529, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytokine receptors; p53; psoriasis; leflunomide

Psoriasis is a common skin disease characterized by epidermal hyperplasia, altered epidermal maturation, and local accumulation of inflammatory cells [1]. All these features may be linked to the dysregulation of growth factors and cytokines and to altered expression of cytokine receptors in involved skin.

Increased expression of IL-8† mRNA has been detected in lesional psoriatic epidermis [2], which suggests a critical role in the pathophysiology of the disease [3]. The effects of IL-8 on keratinocytes are mediated through specific receptors. In lesional psoriatic epidermis, the mRNA level for the IL-8 receptor type A (IL-8RA) are increased approximately 10-fold compared to nonlesional skin [4]. The anti-inflammatory cytokine IL-10 was originally described in Th2 lymphocytes and characterized by its ability to inhibit the typical cytokine response of Th1 cells [5]. IL-10 suppresses the production of proinflammatory cytokines tumor necrosis factor α, IL-1α, IL-1β and IL-6), chemokines (IL-8 and macrophage inflammatory protein 1α), and hematopoietic growth factors (G-CSF) and (GM-CFS) by activated human monocytes [6, 7]. The different activities of IL-10 are mediated via the IL-10 receptor (IL-10R) expressed on the cell surface. So far, no information has been available on the expression of IL-10R in epidermal cells. We observed constitutive expression of IL-10R mRNA in epidermal cells *in vitro* and were subsequently able to show decreased levels of IL-10R mRNA and protein in lesional psoriatic skin [8].

Wild-type p53 is a DNA-binding protein that serves as a negative regulator of the cell cycle. In earlier experiments,

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† *Abbreviations:* A77 1726, N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide; BrdU, 5-bromo-2′-deoxy-uridine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EtBr, ethidium bromide; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-8, interleukin-8; IL-10, interleukin 10; RT-PCR, reverse transcriptase-polymerase chain reaction.

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we observed a down-modulation of p53 transcript levels in psoriatic plaques and an up-regulation of mRNA and protein content in epidermal cells *in vitro* by the immunosuppressive antipsoriatic drug tacrolimus (FK506) [9].

Recently, leflunomide, with A77 1726 being the active metabolite, was shown to be effective in inhibiting reactions leading to organ graft rejection, autoimmune disorders, and human rheumatoid arthritis [10]. A77 1726 exhibits immunomodulatory effects and considerable antiproliferative activity in human lymphocytes [11]. Leflunomide has been shown to be effective and well tolerated in patients suffering from severe rheumatoid arthritis [12]. Its efficacy in psoriasis is currently being evaluated in clinical trials. We investigated the effects of leflunomide's active metabolite, A77 1726, on epidermal cell proliferation and gene expression of pro- (IL-8RA) and anti-inflammatory (IL-10R) mediators as well as the cell-cycle inhibitor p53 [13].

MATERIALS AND METHODS

Drugs and Reagents

A77 1726, a kind gift from Hoechst AG, was diluted in DMEM (Life Technologies) at 25 μM. Taq polymerase and dNTP were obtained from Pharmacia. Superscript RT, oligo-dT-primer, and TRIzol™ were obtained from Life Technologies. Band intensities were quantitated using a densitometric scanner (Elscript 400-AT/SM). The BrdU kit for proliferation assays was obtained from Boehringer Mannheim.

Epidermal Cell Culture

The spontaneously transformed human epidermal cell line HaCaT was cultured at approximately 10^4 cells per well in microtiter plates for 2 days with DMEM containing 10% fetal calf serum (FCS).

Proliferation Assay

The cells were synchronized by serum starvation for 2 days and subsequently incubated in DMEM containing 10% FCS with increasing concentrations of A77 1726 (0–500 μM) for 3 hr, 24 hr, and 48 hr. The proliferation rate was determined by BrdU incorporation into freshly synthesized DNA using a commercial kit (Boehringer Mannheim).

Cytotoxicity

Cells were incubated with A77 1726 (10–100 μM) for 24 hr and washed with PBS before the addition of 5 μg/mL propidiumiodide. Cell viability was assessed by flow cytometry.

RNA Preparation and RT-PCR

Cell cultures were incubated in the presence of 0–50 μM of A77 1726 for 3 hr. The extraction of total RNA was performed according to the TRIzol™ reagent protocol. The quality of the RNA was tested in 1% agarose gels stained with EtBr. The total RNA concentration was measured using a spectral photometer. After RT, gene-specific mRNA levels were determined using semiquantitative RT-PCR. Primer sequences were (L = 5′primer, R = 3′primer):

IL-8RA: L: AAC ACC CTG AGG TTG TGT GT
R: TGG GTT AAA GAT GTG ACG TTG
IL-10R: L: CCA TCT TGC TGA CAA CTT CC
R: GTG TCT GAT ACT GTC TTG GC
β-Actin L: AGA GAT GGC CAC GGC TGC TT
R: ATT TGC GGT GGA CGA TGG AG

Cycling conditions for IL-8RA were: 1.5 min at 94°, 1.5 min at 55°, and 1.5 min at 72° for 1 cycle and then 30 cycles at 94° for 1 min, 55° for 1 min, and 72° for 1 min with a 2-sec time increment per cycle. Cycling conditions for IL-10R and β-actin were: 1.5 min at 94°; 1.5 min at 60°; 1.5 min at 72° for 1 cycle and then 30 cycles at 94° for 1 min, 60° for 1 min, and 72° for 1 min with a 2-sec time increment per cycle. The amplified products were electrophoresed on 2% agarose gels and stained with EtBr. The intensity of PCR products was determined by laser densitometry. The amount of target cDNA was quantitated relative to the β-actin level of each sample.

Northern Blot Hybridization

Ten micrograms total RNA per lane were electrophoresed through a 0.9% agarose-formaldehyde gel, followed by Northern transfer to Hybound-N^+ nylon membrane (Amersham). The membrane was prehybridized in 10 mL of DIG Easy Hyb (Boehringer Mannheim) for 4 hr at 42°, then hybridized with ^{32}P-labeled cDNAs overnight at 42°. p53 and 28S cDNA were from ATCC. After hybridization, the membrane was washed twice with 2 × SSC and 0.1% SDS at 42° for 10 min and once with 0.1 × SSC and 0.1% SDS at 42° for 10 min. The membrane was exposed to Kodak XAR films at −70°. The appropriate bands on the resulting autoradiographs were quantitated by laser densitometry. The amount of target mRNA was quantitated relative to the 28S RNA level of each sample.

Flow Cytometry

IL-10 RECEPTOR. Cells were incubated with A77 1726 (10–100 μM) for 48 hr. After typsinization, cells were allowed to reexpress possibly digested cell surface receptors by transient incubation in media containing the drug. Then, unspecific antibody binding was blocked by a 20-min incubation in 28 μg/mL of human IgG at room temperature. The cells were washed twice in PBS/1% BSA and incubated in 4 μg/200 μL of mouse antihuman-IL-10R IgG

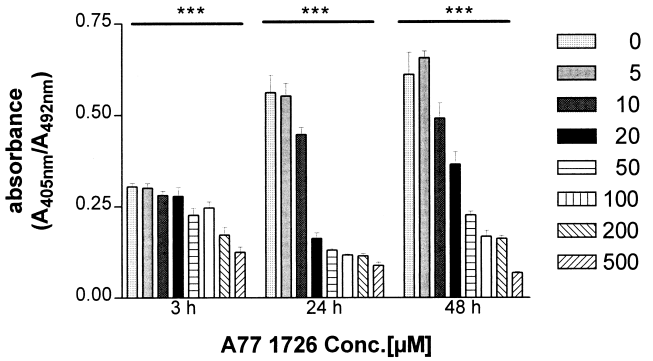

FIG. 1. Effect of A77 1726 on the growth rate of HaCaT cells. Cells were incubated with increasing concentrations of the drug in 96-well plates, and cell proliferation was monitored using a commercial BrdU incorporation kit.The cell proliferation was inhibited dose-dependently by leflunomide after 24 hr and 48 hr.

(a generous gift from Dr. R. deWaal-Malefyt, DNAX Res. Inst., Palo Alto, CA) for 2 hr at room temperature. The second antibody (FITC-conjugated goat antimouse IgG) was added at 2 μg/200 μL and incubated for 90 min. The primary antibody was omitted as negative control.

P53. Cells were incubated with A77 1726 (10–100 μM) for 24 hr. After typsinization, cells were fixed in 4% paraformaldehyde for 20 min at 4° and resuspended in PBS/0. One percent saponin/1% BSA/0.02% NaN_3. FITC-coupled mouse antihuman p53 IgG1 (Camon) was added at a 1:25 dilution and incubated for 1 hr at room temperature. FITC-coupled mouse IgG1 isotype was used as negative control.

RESULTS

Effect of A77 1726 on Cell Proliferation

The DNA synthesis rate was determined by BrdU incorporation. For each concentration, five independent wells were measured. A77 1726 exerted a dose-dependent inhibition of BrdU incorporation at concentrations above 5 μM (Fig. 1). Evaluation of the quintuplicate measurements for each time point separately by one-way ANOVA, including a post-test for linear trend, revealed statistical significance of the effect with a value $P < 0.001\%$.

Effect of A77 1726 on Cell Viability

The drug had no apparent detrimental effect on cell viability as determined by propidium iodide exclusion at concentrations between 10 and 100 μM (Fig. 2).

Influence of A77 1726 on Pro- and Anti-Inflammatory Cytokine Receptor Gene Expression

By using a semiquantitative reverse transcription PCR technique, we analyzed the modulation of gene expression in epidermal cells by A77 1726. Amplification signals for IL-8RA and IL-10R cDNA were densitometrically scanned and normalized to the respective amplification signals of β-actin cDNA. The type A receptor for the proinflammatory and mitogenic cytokine IL-8 was constitutively expressed in HaCaT cells. Treatment with leflunomide's active metabolite A77 1726 caused a dose-dependent down-regulation of IL-8RA mRNA (Fig. 3).

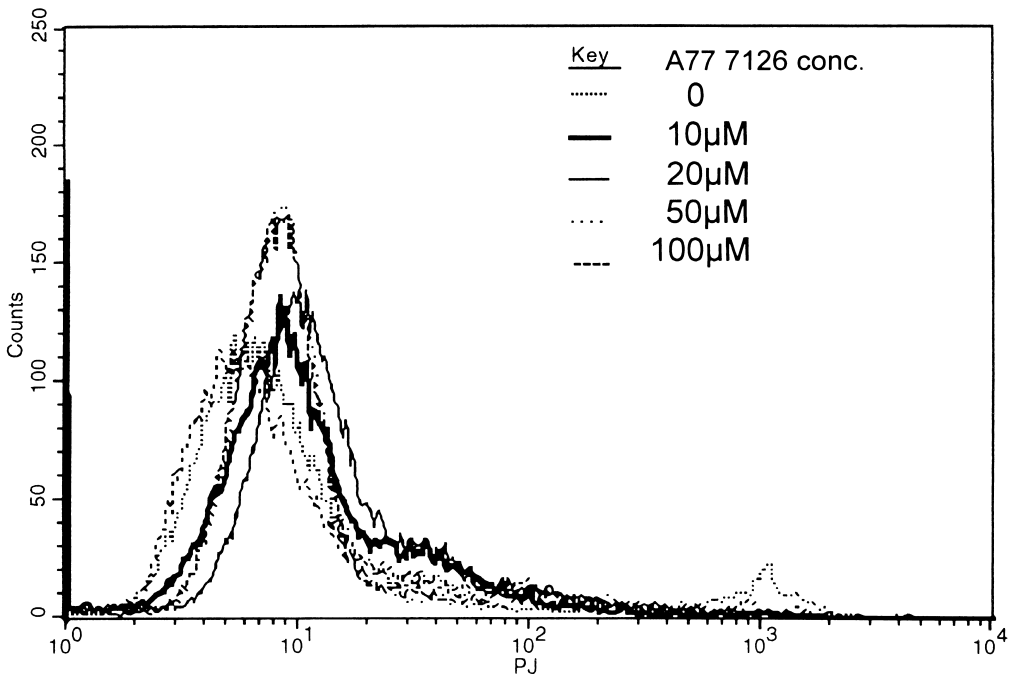

FIG. 2. Effect of A77 1726 on viability of HaCaT cells. Cells were incubated with increasing concentrations of the drug and viability was monitored by flow cytometric evaluation of the dye exclusion capacity of the cells towards propidium iodide. The cells stayed viable even at the highest concentration.

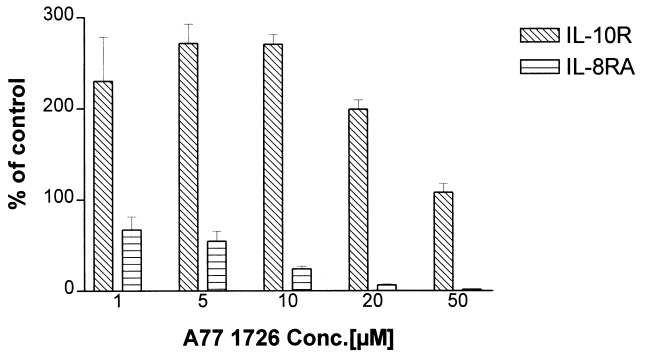

FIG. 3. Effect of A77 1726 on IL-8RA and IL-10R mRNA levels. Semiquantitative evaluation of RT-PCR band intensities by densitometric scanning. Values are normalized to β-actin signals. Control: untreated cells; test cells were treated with the indicated concentrations of A77 7126 for 3 hr. The drug modulated IL-8RA and IL-10R gene expression in opposite directions. Data result from three test series.

IL-10R receptor mRNA was expressed at low levels in HaCaT cells. In contrast to IL-8RA mRNA, A77 1726 caused a 3-fold induction of IL-10R mRNA, with an optimal concentration between 5–10 μM (Fig. 3).

These data on the mRNA level could be substantiated by flow cytometric analysis of the IL-10R protein on the cell surface. To demonstrate integrity of the cells after trypsinization, a foreward/side-scatter plot was recorded (Fig. 4). A77 1726 caused a clear dose-dependent increase of IL-10R-positive cells up to a value of ~20% of the total at the highest concentration, compared with 6% of the total in untreated cells (Fig. 5 and Table 1). The presence of IL-8RA could not be detected by flow cytometry.

Effect of A77 1726 on p53 Gene Expression in HaCaT Cells

Northern blot analysis showed a dose-dependent up-regulation of p53 mRNA in HaCaT cells treated with increasing concentrations of A77 1726 for 3 h. The mRNA level of p53 at a concentration of 50 μM of A77 1726 was induced ~2.5-fold compared with untreated cells (Fig. 6).

This finding on the mRNA level was also confirmed at the protein level by FACS analysis. A77 1726 caused an increase of p53-positive cells up to 76% of the total at 100 μM, compared with 4% of the total in untreated cells (Fig. 7 and Table 2).

DISCUSSION

Psoriasis is characterized by keratinocyte hyperplasia, altered epidermal maturation, and local accumulation of inflammatory cells [1]. Our results demostrate that A77 1726, the active metabolite of leflunomide, a nonsteroidal anti-inflammatory drug with potential use in psoriasis, inhibits the DNA synthesis rate in epidermal cells with an IC_{50} between 10 and 20 μM after 24 hr (Fig. 1). Cytotoxic effects of the substance could be clearly excluded by flow cytometry, which showed that, even at a concentration of 100 μM, virtually all cells stay within the propidium iodide-negative peak area (Fig. 2), indicating that the cells are still vital, but no longer proliferating.

Several lines of evidence point to the possible mechanisms of this growth inhibitory action of A77 1726. Transforming growth factor (TGFα) and receptors of the EGF play an important role in epidermal growth and their intricate regulation seems to be crucial in maintaining the proper balance between growth and differentiation in the

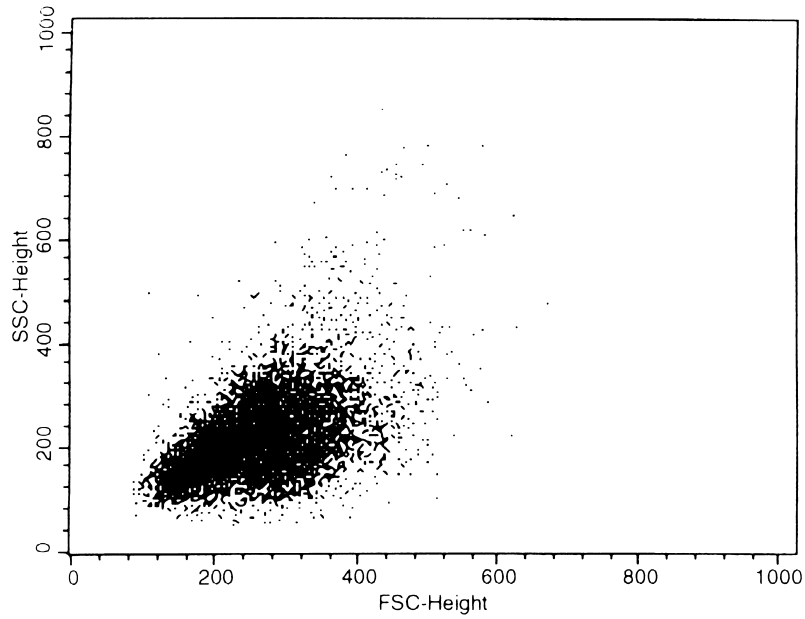

FIG. 4. Cellular integrity. Foreward versus side-scatter dot plot of HaCat cells used for FACS analysis after trypsinization. The cells formed a uniform population.

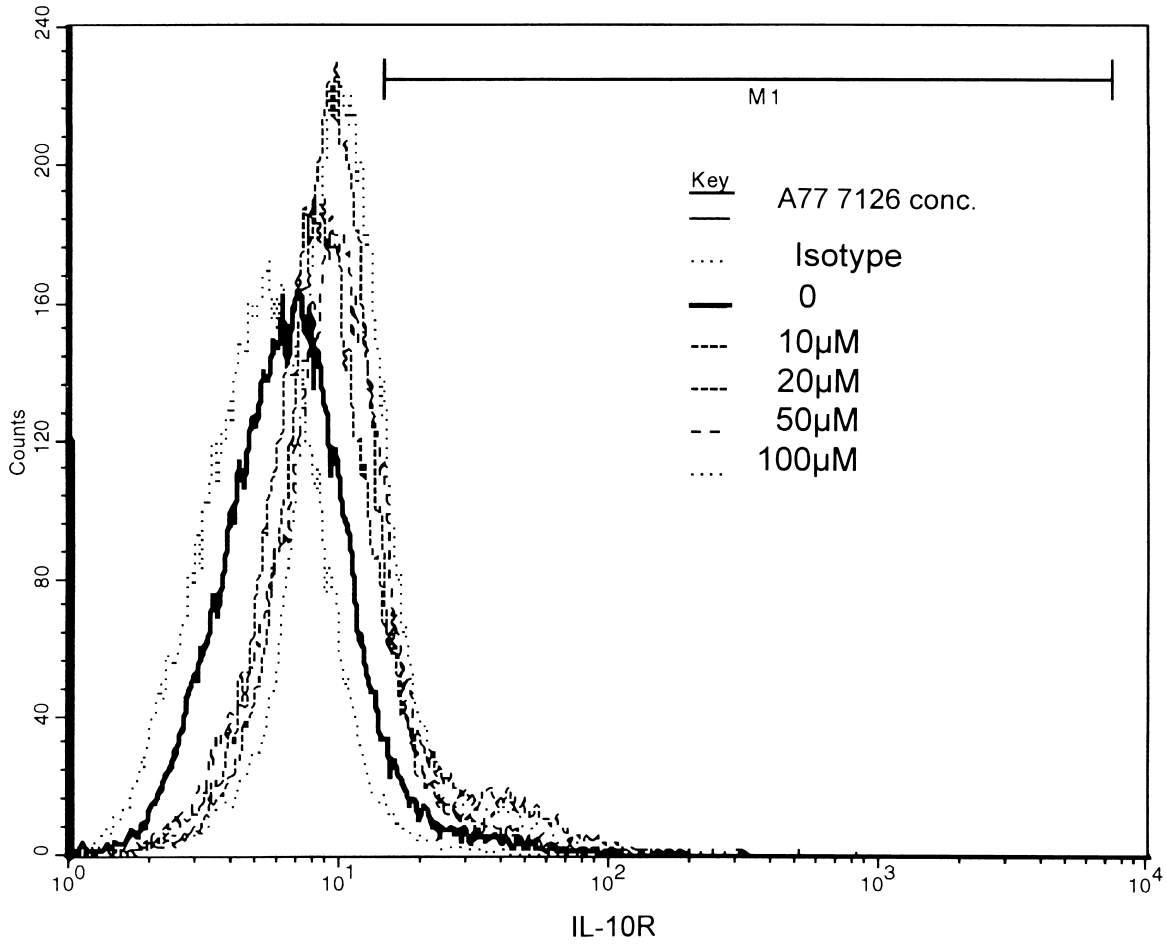

FIG. 5. Effect of A77 1726 on IL-10R protein. Cells were treated with 10–100 μM of the drug and IL-10R expression on the cell surface was determined by flow cytometry. The drug induced IL-10R expression dose dependently.

epidermis [14]. Both growth factors, EGF and transforming growth factor, are ligands for the EGF receptor on the surface of basal epidermal cells [15]. A77 1726 is able to inhibit EGF-dependent cell growth through the inhibition of EGF receptor autophosphorylation at a concentration of 20–30 μM in intact human foreskin fibroblast cells [16]. Moreover, A77 1726 inhibits the EGF receptor tyrosine-specific kinase dose dependently at the same concentration that is required to inhibit fibroblast growth (~40 μM) [16]. These drug levels were the basis for the concentration range used in our experiments. Our results show that A77 1726 inhibits keratinocyte growth at even lower concentrations (10–20 μM) than fibroblast proliferation. Inhibition of EGF receptor autophosphorylation is only one possible explanation for the antiproliferative effect of A77 1726 in keratinocytes. A77 1726 is also able to inhibit dihydroorotate-dehydrogenase (EC 1.3.3.1), an enzyme involved in pyrimidine *de novo* synthesis, at much lower concentrations than is necessary to inhibit tyrosine kinase [17, 18]. However, it remains unclear which, if not both, of these biochemical effects is responsible for the many immunomodulating activities of leflunomide *in vivo*. The evidence speaks for a dual mode of action of leflunomide, namely, interference in signaling (tyrosine kinase) and proliferation (dihydroorotate-dehydrogenase).

TABLE 1. Effect of A77 1726 on IL-10R expression

Drug concentration (μM)	Marker	Events	% Total
Isotype	All	23387	100
	M1	426	2
0	All	22430	100
	M1	1357	6
10	All	22136	100
	M1	2750	12
20	All	23189	100
	M1	4025	17
50	All	21987	100
	M1	3412	15
100	All	13439	100
	M1	4592	20

Dysregulation of numerous genes has been described in the pathophysiology of psoriasis [1]. In earlier studies, our group observed overexpression of IL-8R mRNA [4] and reduced transcript levels of the cell-cycle inhibitor p53 in lesional psoriatic skin [9]. For both genes, the dysregulation

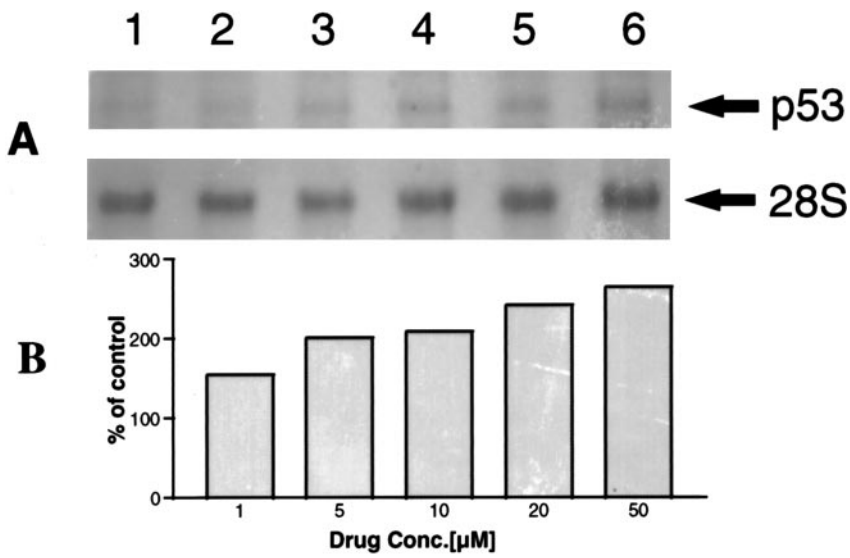

FIG. 6. Effect of A77 1726 on the p53 mRNA level. Gene expression was monitored by Northern blot analysis. A) Lane 1: untreated cells; lanes 2–6: treatment with 1 μM, 5 μM, 10 μM, 20 μM, and 50 μM of A77 1726 for 3 hr. B) Densitometric scanning of band intensities normalized to 28S signals. A77 1726 induced p53 transcription dose-dependently.

could be reversed by treatment with antipsoriatic drugs [4, 9, 19].

Here, we show that A77 1726 is able to inhibit IL-8RA (Fig. 2) and induce p53 (Fig. 3, 6 and Table 2) gene expression dose-dependently *in vitro*. The elevated expression of the cell-cycle inhibitor by A77 1726 is in good concordance with our finding of a decreased growth rate measured by BrdU incorporation as described above and may be a further explanation for the antiproliferative effect of the drug.

In earlier experiments, we were able to show constitutive gene expression of the receptor for the anti-inflammatory and growth inhibitory cytokine IL-10 (IL-10R) *in vitro* and *in situ* and decreased levels of IL-10R mRNA in lesional psoriatic skin [8]. Therefore, the IL-10R gene may be involved in the pathogenesis of psoriasis. Thus, we investigated the modulation of IL-10R gene expression by A77 1726. The drug induced IL-10R transcripts and protein dose-dependently (Fig. 2, 5 and Table 1). This finding supports a model in which psoriatic keratinocytes with decreased IL-10R levels are no longer able to respond to the growth inhibitory action of the ligand IL-10. This response can be restored by treatment with A77 1726.

The difference in the magnitude of induction between the mRNA and protein levels of IL-10R and p53 indicates that both genes are subject to dual regulation at the transcriptional and at the post-transcriptional level.

In summary, the antiproliferative character of A77 1726 and the opposite nature of the transcriptional regulation of pro- and anti-inflammatory cytokine receptors and the

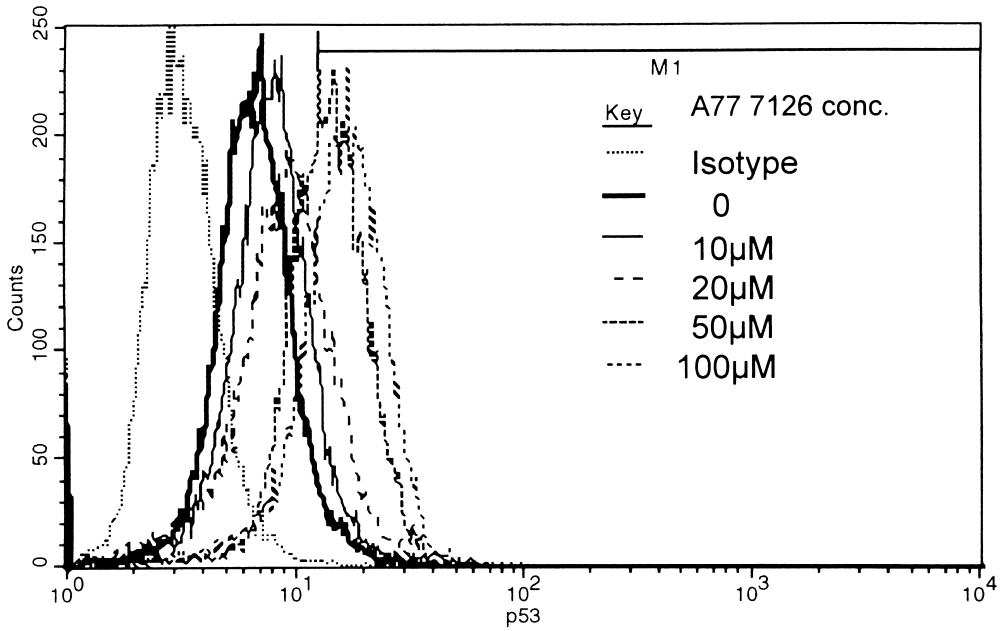

FIG. 7. Effect of A77 1726 on the p53 protein content. Cells were treated with 10–100 μM of the drug and the intracellular p53 content was determined by flow cytometry. A77 1726 induced the p53 protein content dose dependently.

TABLE 2. Effect of A77 1726 on p53 expression

Drug concentration (μM)	Marker	Events	% Total
Isotype	All	20000	100
	M1	24	0.1
0	All	20000	100
	M1	843	4
10	All	20000	100
	M1	1845	9
20	All	20000	100
	M1	4599	23
50	All	20000	100
	M1	12739	63
100	All	20000	100
	M1	15311	76

tumor suppressor gene p53 may contribute to the antipsoriatic effects of A77 1726, the active metabolite of leflunomide.

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