The Tyrosine Kinase Inhibitor Tyrphostin AG126 Reduces the Development of Acute and Chronic Inflammation

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Protein tyrosine kinases help to regulate the expression of many genes that play important roles in inflammation. Here we investigate the effects of the tyrosine kinase inhibitor tyrphostin AG126 in two animal models of acute and chronic inflammation, carrageenan-induced pleurisy and collagen-induced arthritis. We report here that tyrphostin AG126 (given at 1, 3, or 10 mg/kg i.p. in the pleurisy model or 5 mg/kg i.p. every 48 hours in the arthritis model) exerts potent anti-inflammatory effects in animal models of acute and chronic inflammation *in vivo*. These include the inhibition of pleural exudate formation and mononuclear cell infiltration (pleurisy model) and the development of clinical signs and tissue injury (arthritis model). Furthermore, tyrphostin AG126 reduced the staining for nitrotyrosine and poly (ADP-ribose) polymerase (by immunohistochemistry) and the expression of inducible nitric oxide synthase and cyclooxygenase-2 in the lungs of carrageenan-treated rats and in the joints from collagen-treated rats. Thus, we provide the first evidence that prevention of the activation of protein tyrosine kinases reduces the development of acute and chronic inflammation, and that inhibition of the activity of certain tyrosine kinases may represent a novel approach for the therapy of inflammation. (*Am J Pathol* 2000, 157:145–158)

Phosphorylation of proteins on tyrosine residues by protein tyrosine kinases plays an important role in the regulation of cell proliferation, cell differentiation, and signal-

ing processes in cells of the immune system. The receptor tyrosine kinases participate in trans-membrane signaling, whereas the intracellular tyrosine kinases take part in the signal transduction to the nucleus. Enhanced activity of tyrosine kinases has been implicated in the pathophysiology of many diseases associated with local (atherosclerosis, psoriasis) or systemic inflammation, including sepsis and septic shock.¹

An enhanced formation of nitric oxide (NO) due to the expression of the inducible isoform of NO synthase (iNOS) also plays an important role in inflammation.^{2–4} The expression of iNOS caused by inflammatory stimuli in cultured cells involves the phosphorylation of tyrosine residues in proteins and is prevented by the tyrosine kinase inhibitors genistein, erbstatin, and tyrphostin AG126.^{5–9} Activation of tyrosine kinases also mediates the expression of the inducible isoform of cyclooxygenase (COX)-2 caused by endotoxin or pro-inflammatory cytokines in murine macrophages⁷ or in a human epithelial cell line (A549).¹⁰ Induction of COX-2 results in an enhanced formation of metabolites arachidonic acid (eg, vasodilator prostaglandins), which exert pro-inflammatory effects.^{11–13}

We have hypothesized that the inhibition of the activity of protein tyrosine kinases may represent a novel approach for the treatment of acute and chronic inflammation. Many previous strategies aimed at reducing inflammation have been limited to targeting a single mediator in one compartment of the body.^{14–21} In contrast, tyrosine kinase inhibitors act directly on cells, not mediators. Thus, inhibitors of tyrosine kinase activity should reduce the formation and/or effects of pro-inflammatory cytokines, eg, tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1), the expression of iNOS and COX-2, and the activation of the transcription factor nuclear factor- κ B (NF- κ B).^{22–27} Although all of these effects of inhibitors of tyrosine kinase should be anti-inflammatory in nature, there are no studies investigating the effects of tyrosine

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Table 1. Effect of Tyrphostin AG 126 on Carrageenan-Induced Inflammation, Cytokine Levels, and NO Formation in the Pleural Exudate

Treatment group	Volume exudate (ml)	PMNs infiltration (million cells/rat)	TNF- α (pg/ml)	IL-1 β (pg/ml)	Nitrite/nitrate (nmol/rat)	Lung iNOS activity fmoles/mg/minute
Sham + Vehicle	0.13 $\pm$ 0.06	2 $\pm$ 0.8	17 $\pm$ 9	2 $\pm$ 1	6.1 $\pm$ 2	5 $\pm$ 0.8
Sham + AG 126 (1 mg/kg)	0.11 $\pm$ 0.11	1.9 $\pm$ 0.7	15 $\pm$ 7	3 $\pm$ 2	4.9 $\pm$ 1.1	6 $\pm$ 0.7
Sham + AG 126 (3 mg/kg)	0.1 $\pm$ 0.08	1.8 $\pm$ 0.6	18 $\pm$ 9	2.8 $\pm$ 1.2	5 $\pm$ 1.08	1.8 $\pm$ 0.6
Sham + AG 126 (10 mg/kg)	0.16 $\pm$ 0.05	2.3 $\pm$ 0.9	14 $\pm$ 3	3.5 $\pm$ 2.4	3.9 $\pm$ 1.5	2.3 $\pm$ 0.9
CAR + Vehicle	1.3 $\pm$ 0.11*	85 $\pm$ 3.4*	545 $\pm$ 23*	261 $\pm$ 21*	88 $\pm$ 4.3*	150 $\pm$ 3.4*
CAR + AG 126 (1 mg/kg)	0.71 $\pm$ 0.12 [†]	60 $\pm$ 3.2 [†]	300 $\pm$ 29 [†]	105 $\pm$ 15 [†]	55 $\pm$ 2 [†]	100 $\pm$ 3.2 [†]
CAR + AG 126 (3 mg/kg)	0.44 $\pm$ 0.1 [†]	40 $\pm$ 2.9 [†]	219 $\pm$ 45 [†]	84 $\pm$ 13 [†]	32 $\pm$ 2.1 [†]	71 $\pm$ 4.9 [†]
CAR + AG 126 (10 mg/kg)	0.25 $\pm$ 0.09 [†]	25 $\pm$ 3.2 [†]	178 $\pm$ 7 [†]	66 $\pm$ 8 [†]	21 $\pm$ 3.1 [†]	34 $\pm$ 5.1 [†]

Data are means $\pm$ SE of 10 rats for each group.

* $P < 0.01$ versus sham.

[†] $P < 0.01$ versus carrageenan.

kinase inhibitors in animal models of acute and chronic inflammation.

A family of tyrosine kinase inhibitors, the tyrphostins, which are derivatives of benzylidene malononitrile, have recently been discovered. Here we investigated the effects of tyrphostin AG126, a protein kinase inhibitor that prevents the activation of mitogen-activated protein kinase, p42MAPK (ERK2), on acute and chronic inflammation (carrageenan-induced pleurisy and collagen-induced arthritis). In particular, we investigated the effects of tyrphostin AG126 on the lung injury associated with carrageenan-induced pleurisy and the joint injury associated with collagen-induced arthritis. To gain a better insight into the mechanism of action of tyrphostin AG126, we also investigated the effects of tyrphostin AG126 on the expression of iNOS and COX-2 protein (immunohistochemistry) and activity, peroxynitrite formation and activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) by immunohistochemistry.

Materials and Methods

Animals

Male Sprague-Dawley and Lewis rats (160–180 g; Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (Decreto Ministeriale 116192) as well as with the European Economic Community regulations (O. J. of E. C. L 358/1 12/18/1986).

Experimental Groups (Pleurisy Study)

In the treated group of animals, tyrphostin AG126 was given intraperitoneally (i.p.) as a bolus injection at 15 minutes before carrageenan (1, 3, or 10 mg/kg; CAR + AG126 group). In a vehicle-treated group of rats, vehicle

(ethanol, final concentration 1%) was given instead of tyrphostin AG126 (CAR group). In separate groups of rats, surgery was performed in every aspect identical to the one in the CAR group, except that saline was injected instead of carrageenan (sham group; Sham). In an additional group of animals, sham surgery was combined with the administration of tyrphostin AG126 (dose as above; Sham + AG126).

Carrageenan-Induced Pleurisy

Rats were anesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and either saline (0.2 ml) or saline containing 1% λ -carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 hours after the injection of carrageenan, the animals were killed by inhalation of CO₂. The chest was carefully opened and the pleural cavity rinsed with 2 ml of saline solution containing heparin (5 U/ml⁻¹) and indomethacin (10 μ g/ml⁻¹). The exudate and washing solution were removed by aspiration and the total volume measured. Any exudate contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in phosphate-buffered saline (PBS) and counted with an optical microscope in a Burker's chamber after vital Trypan blue staining.

Measurement of Nitrite/Nitrate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in the supernatant samples as previously described.⁴ Briefly, the nitrate in the supernatant was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml⁻¹) and NADPH (160 μ mol/L) at room temperature for 3 hours. The nitrite concentration in the samples

Figure 1. Immunohistochemical localization of iNOS and COX-2 in the lung. Four hours after carrageenan injection, positive staining for iNOS (**A**) and COX-2 (**C**) was localized mainly in macrophages. There was a marked reduction in the immunostainings in the lungs of carrageenan-treated rats pre-treated with tyrphostin AG126 (10 mg/kg; **B** and **D**). Original magnification, $\times 125$. Figure is representative of at least 3 experiments performed on different experimental days.

was then measured by the Griess reaction, by adding 100 μ l of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4 ; vol. 1:1) to 100- μ l samples. The optical density at 550 nm (OD_{550}) was measured using an enzyme-linked immunosorbent assay microplate reader (SLT-Labinstruments, Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of Dulbecco's modified Eagle's medium high glucose.

Induction of Collagen-Induced Arthritis

Bovine type II collagen (CII) was dissolved in 0.01 mol/L acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at $-70^\circ C$ until use. Complete Freund's adjuvant (CFA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. Before injection, CII was emulsified with an equal volume of CFA. Collagen-induced

arthritis was induced as previously described.²⁸ On day 1, Lewis rats were injected intradermally at the base of the tail with 100 μ l of the emulsion (containing 100 μ g of CII). On day 21, a second injection of CII in CFA was administered. In another set of experiments, animals were treated with tyrphostin AG126 ($n = 10$; 5 mg/kg, i.p.) every 48 hours, starting from day 24.

Clinical Assessment of CIA

Rats were evaluated daily for arthritis by using a macroscopic scoring system: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis of the entire paw and digits. Arthritic index for each rats was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile).

Figure 2. Effect of tyrphostin AG126 on nitrotyrosine formation and PARP activation. Four hours following carrageenan injection, positive staining for nitrotyrosine and for PARP was observed (**A** and **C**). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with tyrphostin AG126 (10 mg/kg; **B** and **D**). Original magnification, $\times 125$. Figure is representative of at least 3 experiments performed on different experimental days.

Histological Assessment of Joint Injury

At day 35, animals were sacrificed while they were under anesthesia, and paws and knees were removed and fixed for histological examination, which was done by an investigator blinded to the treatment regimen. The following morphological criteria were considered: 0, no damage; 1, edema; 2, inflammatory cell presence; 3, bone resorption.

Histological Examination

Lung biopsies (4 hours after injection of carrageenan), paws and knees (35 days after CIA). The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in PBS) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). The paws were trimmed, placed in decalcifying solution for 24 hours, embedded in paraffin, and sectioned at 5 μ m. Tissue sections were deparaffinized with xylene, stained with trichromic Van Gieson, and studied using light microscopy (Dialux 22 Leitz).

Radiography

The rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.). Rats were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and arthritic rat hind paws was performed by X-ray machine (Philips X12, Hamburg, Germany) with a 40 kW exposure for 0.01 seconds. An investigator blinded to the treatment regimen performed radiograph scoring. The following radiograph criteria were considered: 0, no bone damage; 1, tissue swelling and edema; 2, joint erosion; 3, bone erosion and osteophyte formation.

Immunohistochemical Localization of Nitrotyrosine

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described.⁴ At the end of the experiment, the relevant organs were fixed in 10% buffered formaldehyde and 8- μ m

Figure 3. PGE₂ levels in the pleural exudate (A) and 6-keto-PGF_{1α} in the lungs (B) from carrageenan-treated rats. The amounts of PGE₂ and 6-keto-PGF_{1α} was significantly reduced in a dose dependent manner in rats treated with tyrphostin AG126 (1, 3, or 10 mg/kg). Data are means $\pm$ SE of 10 rats for each group. **P* < 0.01 versus sham. °*P* < 0.01 versus carrageenan.

sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in 60% methanol for 30 minutes. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin. The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex.

Immunohistochemical Localization of PARP

At the specified time after the carrageenan injection, lung tissues were fixed in 10% buffered formalin and 8- μ m sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in 60% methanol for 30 min-

Figure 4. Effect of tyrphostin AG126 on myeloperoxidase activity and malondialdehyde levels in the lung. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B) in the lungs of carrageenan-treated rats killed at 4 hours. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to sham rats (**P* < 0.01). Tyrphostin AG126 (5, 10, or 20 mg/kg) reduced the carrageenan-induced increase in MPO activity and MDA levels in a dose-dependent manner. Values are means $\pm$ SE of 10 rats for each group. **P* < 0.01 versus sham; °*P* < 0.01 versus carrageenan.

utes. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with 1:500 dilution of primary anti-poly (ADP-Ribose) antibody (DBA) or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA).

Myeloperoxidase (MPO) Activity

MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described.²⁹ At the specified time after the intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each pieces of tissue was homogenized in a

Figure 5. Effect of tyrphostin AG126 on lung injury. The lung section from a carrageenan-treated rat (**A**) demonstrates interstitial hemorrhage and polymorphonuclear leukocyte accumulation. The lung section from a carrageenan-treated rat that had received tyrphostin AG126 (20 mg/kg; **B**) demonstrates reduced interstitial hemorrhage and a lesser cellular infiltration. Original magnification, $\times 62.5$. Figure is representative of at least 3 experiments performed on different experimental days.

solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at $20,000 \times g$ at 4°C . An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mmol/L) and 0.1 mmol/L H_2O_2 . The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading $1 \mu\text{mol}/\text{min}^{-1}$ of H_2O_2 at 37°C and was expressed in milliunits per gram weight of wet tissue.

Malondialdehyde (MDA) Measurement

MDA levels in the lung tissue were determined as an indicator of lipid peroxidation.³⁰ Lung tissues collected at the specified time were homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% sodium dodecyl sulfate, 1500 μl of 20% acetic acid (pH 3.5),

1500 μl of 0.8% thiobarbituric acid, and 700 μl of distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged at $3000 \times g$ for 10 minutes. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Determination of Nitric Oxide Synthase Activity

The calcium-independent conversion of L-arginine to L-citrulline in the homogenates of either pleural macrophages or lungs (obtained 4 hours after carrageenan treatment in the presence or absence of tyrphostin AG126) served as an indicator of iNOS activity.⁴ Cells were scraped into a homogenization buffer composed of 50 mmol/L Tris.HCl, 0.1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4) and homogenized in the buffer on ice using a tissue homogenizer. Conversion of [^3H]-L-arginine to [^3H]-L-citrulline was measured in the homogenates as described.⁴ Briefly, homogenates (30 μl) were incubated in the presence of [^3H]-L-arginine (10 $\mu\text{mol/L}$, 5 kBq per tube), NADPH (1 mmol/L), calmodulin (30 nmol/L), tetrahydrobiopterin (5 $\mu\text{mol/L}$), and EGTA (2 mmol/L) for 20 minutes at 22°C . Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing 2 mmol/L EGTA and 2 mmol/L EDTA. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^3H]-L-citrulline activity was measured by a Beckman scintillation counter.

Measurement of Prostaglandin E_2 in the Pleural Exudate

The amount of prostaglandin E_2 (PGE_2) present in the pleural fluid was measured by radioimmunoassay without prior extraction or purification.³¹

Assessment of COX Activity

Lungs were obtained at 4 hours after the induction of pleurisy by carrageenan injection. The material was homogenized at 4°C in a buffer containing the protease inhibitors 20 mmol/L tampone Hepes, pH 7.2, 320 mmol/L saccharosio, 1 mmol/L dithiothreitol, 10 $\mu\text{g}/\text{ml}$ STY, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin in ratio of 5:1 (v/w). The protein concentration in the homogenates was measured by the Bradford assay,³² with bovine serum albumin (BSA) used as standard. Homogenates were incubated at 37°C for 30 minutes in the presence of excess arachidonic acid (30 $\mu\text{mol/L}$). The samples were boiled and centrifuged at $10,000 \times g$ for 10 minutes. The concentration of 6-keto-prostaglandin- $\text{F}_{1\alpha}$ present in the supernatant was measured by radioimmunoassay as previously described.³³

Immunohistochemical Localization of COX-1 and COX-2

Lung biopsies were fixed in 10% buffered formalin and 8- μm sections were prepared from paraffin-embedded

Figure 6. Effect of tyrphostin AG126 on the onset, the secondary lesion and on body weight gain in collagen-induced arthritis. The percentage of arthritic rats (rats showing clinical arthritis scores >1) are represented (A). There was a significant increase in the arthritic score from day 26 ($P < 0.01$) (B). Beginning on day 25, the collagen-challenged rats gained significantly less weight than the normal rats, and this trend continued through day 35 (C). The swelling in hind paws (D) over time (ml) was measured at 2-day intervals. Tyrphostin AG126 positively affected the percentage of arthritic rats, the arthritic score, and the weight gain, as well as the paw edema of CIA-immunized rats. Values are means $\pm$ SE of 10 animals for each group. * $P < 0.01$ versus control. $^{\circ}P < 0.01$ versus CIA.

tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 minutes. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Nonspecific binding was minimized by incubating the section in 2% normal goat serum in PBS for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin (DBA). The sections were, then, incubated overnight with a 1:500 dilution of the primary anti-COX-1 or anti-COX-2 antibody (DBA) or with control solutions. Controls included buffer alone or nonspecific, purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA; 1:100).

Measurement of Cytokines

TNF- α and IL-1 β levels were evaluated in the exudate at 4 hours after the induction of pleurisy by carrageenan injection and in the plasma from CIA-treated rats. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corp., La Jolla, CA). The enzyme-linked immunosorbent assay has a lower detection limit of 30 pg/ml.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Thiopental sodium (intraval sodium) was obtained from Rhône Mérieux Ltd. (Harlow, UK). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, Primary anti-nitrotyrosine, anti-poly (ADP-Ribose) synthetase antibodies primary anti-iNOS, anti-COX-2 and avidin-biotin peroxidase complex were obtained from DBA. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Health Care Ltd., Thetford, UK).

Statistical Evaluation

All values in the figures and text are expressed as mean $\pm$ SE of the mean of n observations. For the *in vivo* studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one- or two-way analysis of variance, and individual group means were then com-

Figure 7. Representative histology of the inflammatory cells infiltration and bone erosion (**A**) of an arthritic animal. Note the reduction in the degree of inflammatory cells infiltration (**B**) in the paws of the tyrphostin AG126-treated arthritic animals. Original magnifications, $\times 100$. Figure is representative of at least 3 experiments performed on different experimental days.

pared with Student's unpaired *t*-test. For the arthritis studies, Mann-Whitney *U* test (two-tailed, independent) was used to compare medians of the arthritic indices.²⁸ A *P* value < 0.05 was considered significant

Results

Effects of Tyrphostin AG126 in Carrageenan-Induced Pleurisy

All rats treated with carrageenan developed an acute pleurisy, characterized by the production of 1.30 ± 0.09 ml of turbid exudate (Table 1). When compared to the number of cells collected from the pleural space of sham-operated rats ($1.8 \pm 0.6 \times 10^6$ /rat; Table 1), injection of carrageenan induced a significant increase in the number of PMNs ($85 \pm 1.9 \times 10^6$ /rat; Table 1). Pretreatment of rats with tyrphostin AG126 attenuated, in a dose-dependent manner, the volume of the pleural exudate as well as the number of PMNs within the exudate in a dose-related fashion (Table 1). The levels of TNF- α and IL-1 β were significantly elevated in the exudate at 4 hours after carrageenan administration. In contrast, the levels of these cytokines were significantly lower in rats treated with tyrphostin AG126 (Table 1). No significant increase in the levels of cytokines was observed in the exudate of sham-operated rats.

The levels of NO_x were also significantly (*P* < 0.01) increased in the exudate after carrageenan challenge (Table 1). In the lungs obtained from animals subjected to carrageenan-induced pleurisy, a significant increase in inducible nitric oxide synthetase (iNOS) activity was detected at 4 hours after injection of carrageenan (Table 1). Pretreatment of rats with tyrphostin AG126 significantly

reduced, in a dose-dependent fashion, both NO_x levels and iNOS activity (Table 1). Immunohistochemical analysis of lung sections obtained from carrageenan-treated rats revealed a positive staining for iNOS, which was primarily localized in alveolar macrophages (Figure 1A). In contrast, no positive iNOS staining was found in the lungs of carrageenan-treated rats, which had been pretreated with tyrphostin AG126 (10 mg/kg; Figure 1B). Staining was absent in control tissue (data not shown). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan revealed a positive staining for nitrotyrosine, which was primarily localized in alveolar macrophages and in airway epithelial cells (Figure 2A). In contrast, no positive nitrotyrosine staining was found in the lungs of carrageenan-treated rats, which had been pretreated with tyrphostin AG126 (10 mg/kg; Figure 2B). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed a positive staining for PARP (Figure 2C). In contrast, no positive staining for PARP was found in the lungs of carrageenan-treated rats that had been pretreated with tyrphostin AG126 (10 mg/kg; Figure 2D). There was no staining for either nitrotyrosine or PARP in lungs obtained from sham-operated rats (data not shown).

The COX activity in carrageenan-induced pleural exudate and lung homogenates was assessed by measuring the increase in the formation of PGE₂ in the exudate. The amounts of PGE₂ found in the pleural exudate of carrageenan-treated rats was 185 ± 13 pg/rat (*n* = 6; Figure 3A). The amounts of PGE₂ were significantly lower in the exudate obtained from carrageenan-treated rats that had been pretreated with tyrphostin AG126. In lungs from carrageenan-treated rats, the amount of 6-keto-PGF_{1 α} was 235 ± 21 pg/mg of tissue (Figure 3B). The amount of

Figure 8. Effect of tyrphostin AG126 treatment on histological damage score (A) and radiograph score (B). Values are means $\pm$ SE of 10 animals for each group. * $P < 0.01$ versus control. ^o $P < 0.01$ versus CIA.

6-keto-PGF_{1 α} was significantly reduced in the lungs from carrageenan-treated rats that had been pretreated with tyrphostin AG126 (Figure 3B). Immunohistochemical analysis of lung sections obtained from carrageenan-treated rats revealed a positive staining for COX-2, which was primarily localized in alveolar macrophages (Figure 1C). In contrast, no positive COX-2 staining was found in the lungs of from carrageenan-treated rats that had been pretreated with tyrphostin AG126 (Figure 1D). Staining was absent in tissue obtained from sham-operated control animals (data not shown).

COX-1 was also detected by immunohistochemistry analysis in the lung sections obtained from rats treated with carrageenan, but its positive staining kept almost the same as that in the tissue obtained from sham-operated control animals (data not shown). The lungs of carrageenan-treated rats that had been treated with tyrphostin AG126 showed a similar amount of positive staining for COX-1 as those in the carrageenan-treated rats (data not shown).

All rats treated with carrageenan exhibited a substantial increase in the activities of MPO and MDA in the lungs (Figure 4, A and B). Pretreatment of rats with tyrphostin AG126 attenuated the increase in MPO and MDA caused by carrageenan in the lung (Figure 4, A and B). In sham-operated rats, tyrphostin AG126 had no effect on any of the parameters measured (Figure 4, A and B). Histological examination of lung sections of rats treated with

Figure 9. Plasma levels of TNF- α and IL-1 β . Cytokine levels were significantly reduced in the exudate from AG126-treated rats. Data are means $\pm$ SE of 10 rats for each group. * $P < 0.01$ versus sham. ^o $P < 0.01$ versus CIA.

carrageenan showed edema and tissue injury as well as infiltration of the tissue with PMNs, lymphocytes, and plasma cells (Figure 5A). Tyrphostin AG126 reduced both the lung injury as well as the infiltration of the tissue with white blood cells (Figure 5B).

Effects of Tyrphostin AG126 in Collagen-Induced Arthritis

CIA developed rapidly in rats immunized with CII and clinical signs (periarticular erythema and edema) of the disease (Figure 6A) first appeared in the hind paws between 24 and 26 days post-challenge. Furthermore, a 100% incidence of CIA was observed by day 27 in CII-immunized rats. Neither the clinical signs nor the histopathological features of CIA were observed in rat forepaws during the 28-day evaluation period. The maximum incidence of CIA in the rats treated with tyrphostin AG126 during the 35-day study period was 65% (Figure 6A; $P < 0.05$).

Hind paw erythema and swelling increased in frequency and severity in a time-dependent manner, with maximum arthritis indices of approximately 13 observed between 28 to 25 days post immunization (Figure 6B). Tyrphostin AG126 caused a significant ($P < 0.01$) suppression of the arthritis index between days 25 and 35 post-CII immunization (Figure 6B). There was no macroscopic evidence of either hind paw erythema or edema in the normal control rats (Figure 6B).

The data in Figure 6D demonstrate a time-dependent increase in hind paw (each value represents the mean values of both hind paws) volume (ml) in rats immunized with CII. Maximum paw volume occurred by day 28 in the CII-immunized rats. Tyrphostin AG126 (see Figure 10) significantly ($P < 0.001$) suppressed hind paw swelling from day 24 to 35 post-immunization. A maximal reduction in response hind paw swelling of 60% was observed from day 28 to 35. No increase in hind paw volume over time was observed in normal rat (Figure 6D).

The rate and the absolute gain in body weight were comparable in normal Lewis rats and CII-immunized rats

Figure 10. Radiographic progression of CIA in the tibiotarsal joint of rats with CIA. There is no evidence of pathology in the tibiotarsal joints of normal rats (**A**). The hind paws from CII-immunized (35 days) rats demonstrated bone resorption (**arrow, B**). Tyrphostin AG126 suppressed joint pathology and soft tissue swelling in the rat hind paw (**C**). Figure is representative of at least 3 experiments performed on different experimental days.

for the first week (Figure 6C). Beginning on day 25, the collagen-challenged rats gained significantly less weight than the normal rats, and this trend continued through day 35. After administration of tyrphostin AG126, CII-immunized rats exhibited a significant ($P < 0.001$) weight gain when compared with the respective control group.

At day 35, the histological evaluation of the paws of vehicle-treated arthritic animals revealed signs of severe arthritis, with massive mixed (neutrophil, macrophage, and lymphocyte) infiltration. In addition, severe or moderate necrosis and sloughing of the synovium were seen, together with the extension of the inflammation into the adjacent musculature with fibrosis and increased mucus production (Figure 7A; see Figure 8A for damage score). In the animals treated with tyrphostin AG126, the degree of arthritis was significantly reduced (Figures 7B and 8A).

A radiographic examination of hind paws from rats 35 days post-CII immunization revealed bone matrix resorption and osteophyte formation at the joint margin (Figure 9B; see Figure 8B for radiograph score). There was no evidence of pathology in normal rats (Figures 8B and 9A). Tyrphostin AG126 markedly reduced the degree of bone resorption, soft tissue swelling, and osteophyte formation (Figures 8B and 9C).

At day 35, the levels of $\text{TNF}\alpha$, and $\text{IL-1}\beta$ were significantly elevated in the plasma from CIA-treated rats. In contrast, the levels of these cytokines were significantly lower in rats treated with tyrphostin AG126 (Figure 10). No significant increase in the levels of cytokines was observed in the plasma of sham-operated rats.

A substantial increase in the plasma MDA levels was found at day 35 in all vehicle-treated arthritic animals (Figure 11). Treatment of rats with tyrphostin AG126 significantly attenuated the increase in MDA caused by

CIA-induced arthritis (Figure 11). No increases in plasma MDA levels were observed in normal rats (Figure 11).

Immunohistochemical analysis of joint sections obtained from rats treated with collagen type II revealed a positive staining for nitrotyrosine, which was primarily localized in synovia portion (Figure 12A). In contrast, no positive nitrotyrosine staining was found in the joint of CIA-treated rats, which had been pre-treated with tyrphostin AG126 (Figure 12B). Immunohistochemical analysis of joint sections obtained from rats treated with collagen type II also revealed a positive staining for PARP (Figure 12C). In contrast, no specific positive staining for PARP was found in the joint of CIA-treated rats, which had been pretreated with tyrphostin AG126 (Figure 12D). Please note that there was no staining for either nitrotyrosine or PARP in joint obtained from sham-operated rats (Data not shown).

Immunohistochemical analysis of joint sections obtained from rats treated with collagen type II revealed a positive staining for iNOS and COX-2 (Figure 13, A and C). In contrast, no positive iNOS and COX-2 staining was found in the joints of CIA-treated rats pretreated with tyrphostin AG126 (Figure 13, B and D).

Discussion

The inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF), and interleukin-1 (IL-1) and by a release of chemicals from tissues and migrating cells.³³ Furthermore, there is a large amount of evidence that the production of reactive oxygen species (ROS) such as hydrogen peroxide, super-

Figure 11. Effect of tyrphostin AG126 on malondialdehyde levels in the plasma: Malondialdehyde (MDA) levels in the plasma of CII-immunized rats killed at 35 days. MDA levels were significantly increased in the plasma of the CII-immunized rats in comparison to sham rats (* $P < 0.01$). Tyrphostin AG126 reduced the CIA increase in MDA levels. Values are means $\pm$ SE of 10 rats for each group. * $P < 0.01$ versus control. ° $P < 0.01$ versus CIA.

oxide, and hydroxyl radicals at the site of inflammation contribute to tissue damage.^{4,26,34–36} Inhibitors of NOS activity reduce the development of carrageenan-induced inflammation and support a role for NO in the pathophysiology associated with this model of inflammation.^{4,36–39} In addition to NO, peroxynitrite is also generated in carrageenan-induced inflammation.^{4,35–37} The biological activity and decomposition of peroxynitrite is very dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, carbon dioxide levels, and other factors), and these factors influence its toxic potential.⁴⁰

This study provides the first evidence that pretreatment of rats with tyrphostin AG126 attenuates (i) the development of carrageenan-induced pleurisy, (ii) the infiltration of the lungs with PMNs (histology and MPO activity), (iii) the degree of lipid peroxidation in the lung, (iv) the degree of lung injury (histology) caused by injection of carrageenan, (v) the development of collagen-induced arthritis, (vi) the infiltration of the joint with PMNs (histology), (vii) the degree of plasma lipid peroxidation, and (viii) the degree of joint injury (histology, radiography) in rats treated with type II collagen. All of these findings support the view that the protein tyrosine kinase inhibitor tyrphostin AG126 attenuates the degree of acute and chronic inflammation in the rat.

What, then, is the mechanism by which tyrphostin AG126 protects the joint against this inflammatory injury? Tyrphostin AG126 is a derivative of benzylidene malononitrile, which is a potent inhibitor of protein tyrosine kinases *in vitro* and *in vivo* (see Introduction), which, among other effects, reduces the biosynthesis and/or the effects of the pro-inflammatory cytokines TNF- α and IL-1. There is good evidence that TNF- α and IL-1 help to propagate the extension of a local or systemic inflammatory process.^{41–44} We confirm that the models of acute and chronic inflammation used here, namely pleurisy and collagen-induced arthritis, lead to a substantial increase in the levels of TNF- α and IL-1 in the exudate and in the

plasma, respectively. Interestingly, the levels of these two pro-inflammatory cytokines are significantly lower in the animals treated with tyrphostin AG126.

Pro-inflammatory cytokines (eg, TNF- α and IL-1) activate the transcription factor NF- κ B, which, in turn, causes the expression of many pro-inflammatory proteins including iNOS, COX-2, TNF- α , IL-1 β , and IL-6, or of the adhesion molecules ICAM-1, VCAM-1, and E-selectin. In the rat, carrageenan and CIA cause an overproduction of NO due to induction of iNOS,^{4,35,37,45} which contributes to the inflammatory process.^{4,35–37,45} We demonstrate here that tyrphostin AG126 attenuates the expression of iNOS in the lung from carrageenan-treated rats (Figure 1B) and in the joints from collagen-treated rats (Figure 13B). Thus, the reduction of the expression of iNOS by tyrphostin AG126 may contribute to the attenuation by this agent of the formation of nitrotyrosine in the lung from carrageenan-treated rats (Figure 2B) and in the joints from collagen-treated rats (Figure 12B). Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation “footprint” of peroxynitrite.⁴⁶ There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; eg, the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine.⁴⁷ Increased nitrotyrosine staining is considered, therefore, as an indication of increased nitrosative stress rather than a specific marker of the generation of peroxynitrite. Thus, we propose that the reduction of the expression of iNOS protein and activity caused by AG126 contributes to the reduction by this agent of the organ injury caused by acute and chronic inflammation in the rat.

We also demonstrate that the increase in the levels of PGE₂ caused by injection of carrageenan into the pleural cavity is reduced in the exudate of rats treated with tyrphostin AG126. The enhanced formation of PGE₂ is secondary to the expression of COX-2 protein, as (i) there was no increase in the expression of COX-1 protein detected by immunohistochemistry after carrageenan injection, and (ii) selective inhibitors of COX-2 activity including NS-398 (nimesulide) and SC-58125 (Celecoxib) markedly abolished the increase in PGE₂ caused by injection of carrageenan into the pleural space.⁴⁸ Thus, we propose that tyrphostin AG126 reduces the expression of COX-2 protein and activity caused by injection of carrageenan in the lung and in the joints from collagen-treated rats.

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARP resulting in the depletion of its substrate nicotinamide adenine dinucleotide (NAD) *in vitro* and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed the PARP suicide hypothesis. There is recent evidence that the activation of PARP may also play an important role in

Figure 12. Nitrotyrosine and PARP immunostaining in the paw of a rat at 35 days of collagen-induced arthritis (**A** and **C**). A marked increase in nitrotyrosine and PARP stainings is evident in the paws in arthritis. There was a marked reduction in the immunostaining in the paw of tyrphostin AG126-treated rats (**B** and **D**). Original magnification, $\times 125$. Figure is representative of at least 3 experiments performed on different experimental days.

inflammation.^{28,34,49} We demonstrate here that tyrphostin AG126 attenuates the increase in PARP activity in the lung from carrageenan-treated rats (Figure 2D) and in the joints from collagen-treated rats (Figure 12D).

In conclusion, this study demonstrates for the first time that the degree of acute and chronic inflammation is significantly attenuated by the protein tyrosine kinase inhibitor tyrphostin AG126. The mechanisms of the anti-inflammatory effects of tyrphostin AG126 are not entirely clear. Clearly, tyrphostin AG126 reduces the recruitment of neutrophils, the expression of iNOS and COX-2 protein and activity, peroxynitrite formation, PARP activation, and, ultimately, tissue injury. One other recent study has demonstrated that the tyrosine kinase inhibitor tyrphostin AG490 exerts anti-inflammatory effects in a model of

autoimmune encephalomyelitis.⁵⁰ As there is little information about the specificity and selectivity toward different protein tyrosine kinases of tyrphostin AG126 (or tyrphostin AG490), it is difficult to pinpoint which of the many known tyrosine kinases play a crucial role in the pathophysiology of inflammation. Although tyrphostin AG126 has been shown to inhibit p42 MAP kinase, it is also possible that tyrphostin AG126 attenuates the activation of the p38 MAP kinase pathway, which plays an important role in acute and chronic inflammation. Further studies aimed at identifying the tyrosine kinase(s) inhibited by tyrphostin AG126 are warranted. Nevertheless, our results support the view⁵⁰ that inhibitors of (some) protein tyrosine kinases, including tyrphostin AG126, may be useful in the treatment of acute and chronic inflammation.

Figure 13. iNOS and COX-2 immunostaining in the paw of a rat at 35 days of collagen-induced arthritis (A and C). A marked increase in iNOS and COX-2 stainings is evident in the paws in arthritis. There was a marked reduction in the immunostaining in the paw of tyrphostin AG126-treated rats (B and D). Original magnification, $\times 125$. Figure is representative of at least 3 experiments performed on different experimental days.

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