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Inhibition of leukocyte elastase, PMN chemoinvasion, and inflammation-triggered pulmonary fibrosis by a 4-alkyliden-β-lactam with a galloyl moiety

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Running title page

a) running title galloyl-β-lactam inhibits elastase and inflammation

(51 characters, including spaces)

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text pages	27
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d) non-standard abbreviations

ECM = extra-cellular matrix

fMLP = N-Formyl-Met-Leu-Phe

MMPs = matrix metallo-proteinases

PMNs = polymorphonuclear leukocytes

TIMPs = tissue inhibitors of MMPs

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Abstract

B-lactams – well known class of antibiotics – have been investigated as inhibitors of the disruptive protease released by inflammatory cells, leukocyte elastase (LE). We have synthesized a new β-lactam with an *N*-linked galloyl-moiety, the latter identified as strategic in conferring anti-LE properties to some flavanols. This N-galloyl-derivative β-lactam inhibits the LE activity with a Ki of 0.7 μM, while exerting week activity against cathepsin G and protease-3 (IC_{50} > 100 μM), and metallo-proteases MMP-2 and MMP-9. Without affecting chemotactic response and viability of polymorphonuclear (PMN) leukocytes, the compound efficiently restrains their chemoinvasion (IC_{50} 1-2 μM) blocking the LE-triggered activation of pro-MMP-9, instrumental to extravasation. Daily *i.p*. injection of compound enhances resolution in a pulmonary inflammation model, significantly reducing consequent fibrosis. These results indicate that the new β-lactam is a potent anti-inflammatory compound with therapeutic potential.

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Introduction

Leukocyte elastase (LE) is a serine protease, expressed by polymorphonuclear (PMN) leukocytes, mainly neutrophils, which acts both intra-cellularly to kill engulfed pathogens, and extra-cellularly as mediator of coagulation, immune responses, and wound debridement (Sternlicht and Werb, 1999). Since LE has the potential to degrade some structural proteins of the extra-cellular matrix (ECM), such as elastin, fibronectin and collagens, excess of LE activity has been involved in a number of pathological conditions leading to impairment of ECM organization, including rheumatoid arthritis, emphysema, cystic fibrosis, and tumor progression (Balckwill and Mantovani, 2001). LE also activates the pro-enzymatic form of matrix metallo-proteinase-9 (MMP-9) (Sternlicht and Werb, 1999) massively released by the PMNs, and instrumental to their extravasation (Delclaux et al., 1996; Esparza et al., 2004).

Human tissues are protected from excessive LE activity by endogenous inhibitors – such as α1-protease-ihibitor (α1-PI), α2-macroglobulin (Lee and Downey, 2001), and secretory leukoprotease inhibitor (Rice and Weiss, 1990) – but any enzyme/inhibitor imbalance may lead to increased lysis of ECM macromolecules and increased risk of tissue injury in areas infiltrated by activated PMNs (Lee and Downey, 2001). Furthermore – given the LE ability to degrade multiple cytokines, receptors, and complement components – a negative modulation of the inflammatory response may favor antigen persistence, leading to chronic inflammation.

As for the possibility of using exogenous LE-inhibitors for therapeutic purposes, most of the inhibitors so far developed cause side effects that make them unsuitable for human use (Teshima et al., 1982). Recently, a number of β-lactams – compounds widely used as antimicrobial drugs – have been identified as inhibitors of serine-enzymes, in particular LE (Konaklieva, 2002). A core structure of four-membered cyclic amide (β-lactam or azetidin-2-one) is the common feature of these molecules; the first LE-inhibitor β-lactams were natural-occurring bicyclic compounds, such as clavams and cephalosporins (Knight et al., 1992), but more recently synthetic monocyclic β-lactams have been developed. Since the latter perform with extremely good safety profiles and infrequent side effects (Kuhn et al., 2004), they could represent a good model-base for designing powerful drugs able to inhibit LE, and restore the altered protease/anti-protease ratio at the inflammatory sites.

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We have already shown that monocyclic β-lactams substituted on C-3, C-4, and N-1 are the most active in inhibiting LE and gelatinases MMP-2 and MMP-9 (Cainelli et al., 2003); and have also reported that C-4 unsaturation on the β-lactam ring rises the inhibitory activity toward these proteases, with selectivity over LE by 3-[1-(*tert*-butyldimethylsilyloxy)-ethyl] derivatives, and over the gelatinase MMP-2 by C-3-unsubstituted 4-[1-ethoxycarbonyl]-ethylidene-β-lactams.

Some catechins (vegetable secondary metabolites of the flavonoid family), and in particular those with a galloyl group (Fig.1B), have been shown to exert a very powerful inhibition of LE activity (Garbisa et al., 2000; Sartor et al., 2002 a; Sartor et al., 2002 b), but their absorption, bioavailability and metabolic fate await full clarification. We have thus synthesized and tested a number of monocyclic β-lactam derivatives with a galloyl-moiety-like group in different positions (Cainelli et al., 2005); some of these – such as the {3-[1-(tert-Butyl-dimethyl-silanyloxy)-ethyl]-4-oxo-1-(3,4,5-tris-benzyloxy-benzoyl)-azetidin-2-ylidene}-acetic acid ethyl ester (Fig.1C) – exert indeed an improved anti-LE activity. Here we show that this latter compound wields the most potent inhibition of LE so far reported for its category, and restrains PMN chemoinvasion, protecting against inflammatory events taking place in the bleomycin-triggered animal model of lung fibrosis.

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Methods

Materials - Elastase (LE) and Proteinase-3 (PR-3) from human leukocytes were obtained from EPC (Elastin Products Company Inc., Owensville, MO, USA); cathepsin G, elastase substrate N-MO-AAPV-p-NA, cytochalasin B, and formyl-Met-Leu-Phe (fMLP) from Sigma Chemical Co.(St. Louis, MO, USA); PR-3 substrate MeO-suc-K(Pic)APV-pNA from Bachem AG (Bubendorf, Switzerland); cathepsin G substrate suc-AAPF-*p*NA from Calbiochem-Novabiochem (Nottingham, United Kingdom); fetal Calf Serum (FCS) from Biochrom (Berlin, Germany); basement membrane matrix (Matrigel®) from Becton Dickinson (Bedford, MA, USA); polyvinilpyrrolidone-free polycarbonate filters from Millipore (St. Louis, MO, USA); and Ficoll-Paque™ Plus (<0.12 EU/ml) from Amersham-Biosciences (Milano, Italy). All of the other reagents, if not specified, were purchased from Sigma.

Synthesis and characterization of β-lactams - The {3-[1-(tert-Butyl-dimethyl-silanyloxy)-ethyl]-4-oxo-azetidin-2-ylidene}-acetic acid ethyl ester (hereafter named "4-alkyliden-β-lactam", compound **2** in Fig.1A) and its derivative {3-[1-(tert-Butyl-dimethyl-silanyloxy)-ethyl]-4-oxo-1-(3,4,5-tris-benzyloxy-benzoyl)-azetidin-2-ylidene}-acetic acid ethyl ester (hereafter named *N*-galloyl-4-alkyliden-β-lactam, compound **3** in Fig.1C) – obtained by inserting a galloyl moiety with O-benzyl protections at the nitrogen atom – were prepared according to the procedure already reported (Prasad et al., 1982) and here out-lined.

Commercial reagents were used as received without additional purification. Anhydrous solvents were obtained commercially and used without further drying. ^{1}H- and ^{13}C-NMR values were recorded on a VARIAN Mercury 400, INOVA 300 or GEMINI 200 instrument using a 5 □m probe. All chemical shifts have been quoted relative to deuterated solvent signals, δ in ppm, *J* in Hz. FT-IR : Nicolet 205 measured as films or nujol mull between NaCl plates and reported in cm^{-1}. TLC: Merck 60 F_{254}. Column chromatography: Merck silica gel 200-300 mesh. HPLC-MS: HPLC : Agilent Technologies HP1100, column ZOBRAX-Eclipse XDB-C8 Agilent Technologies. The products were eluted with CH_3CN/H_2O, gradient: from 30% to 80% of CH_3CN. MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full-scan mode from *m/z* 50 to *m/z* 2600, scan time 0.1 sec in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35 psgi, drying gas flow 11.5 ml/min, fragmetor voltage 20 V. The $[\alpha]^{25}_{D}$ were

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determined with a Perkin Elmer 343 polarimeter Compounds **1** and **2** (Fig.1A) were prepared according to the procedure already reported (Prasad et al., 1982).

Compound **3** [3-[1-(tert-Butyl-dimethyl-silanyloxy)-ethyl]-4-oxo-1-(3,4,5-tris-benzyloxy-benzoyl)-azetidin-2-ylidene]-acetic acid ethyl ester was prepared as follows (Fig.1C): compound **2** (0.381 g, 1.017 mmol) and 3,4,5-tribenzyloxybenzoyl chloride (0.513 g, 1.119 mmol) were dissolved in anhydrous acetone (10 mL); K_2CO_3 (0.141 g, 1.017 mmol) was added and the reaction mixture was stirred at room temperature for 4 hours. Then K_2CO_3 was filtered, the solvent was removed and the crude oily residue was immediately purified by flash chromatography (cyclohexane/ethylacetate = 95/5) to give compound **3**, yield 44%, pale yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.17 (s, 3H), 0.19 (s, 3H), 0.96 (s, 9H), 1.26 (d, *J* = 6.3 Hz, 3H), 1.35 (t, *J* = 7.2 Hz, 3H), 4.25 (q, *J* = 7.2 Hz, 2H), 4.34 (dd, *J* = 4.8 Hz, *J* = 1.2 Hz, 1H), 4.82 (m, 1H), 5.20 (s, 6H), 6.66 (d, *J* = 1.2 Hz, 1H), 7.29-7.48 (m, 17H). ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ -4.9, -4.6, 14.3, 17.9, 19.8, 25.7, 60.3, 64.1, 65.4, 71.4, 75.2, 100.5, 109.9, 125.8, 127.4, 127.5, 128.0, 128.2, 128.4, 128.5, 136.6, 137.3, 143.3, 149.3, 152.4, 164.1, 164.6, 166.2. $[\alpha]^{25}_D$ = +47.3 ($CHCl_3$, c = 4.16). IR (film): 2930, 1841, 1718, 1683, 1328, 1197 cm^{-1}. HPLC: R_t = 9.64 min.

The *in vitro* antioxidant activity of the *N*-galloyl-4-alkyliden-β-lactam was evaluated by the TEAC method (Miller et al., 1993), a spectrophotometric measurement of anti-oxidant-triggered discoloration of a green solution containing a preformed organic cation radical.

Neutrophil isolation - Neutrophils were isolated under endotoxin-free conditions from buffy coats of healthy donors, according to a separation procedure as previously described in detail (Dri et al., 1999). Briefly, freshly prepared buffy coats were diluted 1:3 in PBS, and centrifuged at 180 g for 10 min 3 times to reduce contamination by platelets. After further rinsing in PBS, cells were layered on Ficoll™ density gradient, and centrifuged at 280 g for 30 min. Peripheral blood mononuclear cells were collected at the interface, and PBS and residual Ficoll™ removed. The pellet, which contained erythrocytes and PMN leukocytes, was diluted with 3 volumes of ammonium chloride lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) and incubated at 4 °C for 10 min. After centrifugation (180 g for 10 min, at 4 °C) the lysis was repeated to eliminate residual erythrocytes. The final pellet, assessed by direct observation of nuclear morphology to consist of >96% PMN, was immediately used for the assays, after dilution in the proper buffer solution.

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Inhibition of proteolytic activities - Concentrated stock solutions of LE and cathepsin G were prepared in Hepes buffer (0.1 M Hepes, 0.5 M NaCl, 10% DMSO), PR-3 in Glycine buffer (0.1 M glycine, 0.1 M NaCl), at pH 7.8, 7.5, and 3.2, respectively, as already described (Sartor et al., 2002a; Pezzato et al., 2003). *N*-galloyl-4-alkyliden-β-lactam and substrates were freshly prepared 20x in DMSO, and dilutions of the β-lactam were premixed with the enzyme in 96-well plate, and maintained 30 min at 4 ºC. Then, 5 µl of substrate (8 mM for LE, 10 mM for cathepsin G, 4 mM for PR-3) were added to 100 µl final volume, and the mixture was incubated at 37 ºC. At 20-min intervals, the intensity of the color developed by the digested substrate was measured at 405 nm using a Titertek Mutiskan (Flow Laboratories), and the control background subtracted (in triplicate experiments). The reactions developed linearly for as long as 120 min; data from 60 min were used for plotting the colorimetric reactions. Double-reciprocal plot of the results allowed the type and Ki of inhibition exerted over LE to be deduced.

MMP-9 activation by LE and PMNs - Serum-less medium (Dulbecco's Modified Eagle Medium, DMEM) conditioned 90 min by PMNs under gentle shaking was used as a source of gelatinases, being pro-MMP-9 the prevalent type. LE and *N*-galloyl-4-alkyliden-β-lactam were diluted in DMEM to 1 mU/ml and 2 mM, respectively (the pH of the medium was pre-adjusted to 7.8, to optimize the LE activity and preserve the β-lactam stability); 5 µl LE was mixed with β-lactam solution at 4 °C, and after 15 min the medium was added. Following 1 h incubation at 37 °C, SDS-electrophoresis buffer 4x was added, and the samples processed for 6% polyacrylamide gelatin-zymography (see below).

Gelatin-zymography - Without heating the samples, zymography was performed by electrophoresing the samples in 0.1% gelatin-containing 6% polyacrylamide, in presence of sodium dodecyl sulfate (SDS). After electrophoresis, the gels were washed twice for 30 min with 2.5% Triton X-100, and incubated overnight at 37 ºC in Tris buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4).

For gelatinase inhibition assays, the *N*-galloyl-4-alkyliden-β-lactam was freshly solubilized in DMSO and diluted (1, 10 and 100 µM) in the Tris buffer used for developing separate slices of gelatinase zymograms, which were incubated as above.

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The gels were then stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250, and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis. Digestion bands were quantitated using an image analyzer system with Gel-Doc 2000 and QuantityOne software (Bio-Rad).

PMN chemotaxis, chemoinvasion, and viability - The chemotaxis and chemioinvasion of PMNs in response to fMLP was tested using the modified Boyden chamber assay, as already reported (Benelli et al., 2000). Gelatin (non-barrier for chemotaxis) and Matrigel (barrier for chemoinvasion) were used as matrix substrate for cell migration toward a chemoattractant represented by 10^{-7} M solution of fMLP in DMEM without phenol-red (control experiments were performed in absence of chemoattractant). Polyvinylpyrrolidone-free polycarbonate filters (5-μm pore size) were all pre-coated by immersion in gelatin solution (0.1%) and part over-coated with 50 μl of 0.66 mg/ml Matrigel. After seeding $2x10^6$ cells onto the filters, and 45 min (chemotaxis) or 2 h (chemoinvasion) incubation in serum-free medium with or without the *N*-galloyl-4-alkyliden-β-lactam, non-migrated cells were removed from the upper surface of the filters, and those that actively migrated to the lower chamber harvested and directly counted using a haemocytometer. The results of quintuplicate experiments were averaged after background subtraction (control experiments).

The effect of the *N*-galloyl-4-alkyliden-β-lactam on short-term PMN-viability was also verified. Freshly isolated PMNs were seeded ($5x10^5$) onto 96-well plates, and incubated in 5% CO_2 air at 37 ºC in 90 μl of DMEM without phenol red, supplemented with or without 10% FCS, and with or without the β-lactam at increasing concentrations. After 4 and 20 h, the cell viability was determined by CellTiter 96® assay (Promega).

Bleomycin-induced pulmonary fibrosis - Ten to twelve week-old C57BL6/N mice (weighing 22-25 grams) from Charles River (Lecco, Italy) were maintained 14 days in a germ-free environment and allowed free access to food and water before use. Then, pulmonary fibrosis was induced in 32 animals by intra-tracheal instillation of bleomycin as previously described (Keane et al., 1999). Here, bleomycin was dissolved to a concentration of 1,25 U/ml in sterile PBS, and vortexed extensively, before each 100-μl aliquot (5U/Kg) was used. The mice were anesthetized, the trachea was exposed and entered with a 29-gauge needle, intra-tracheal instillation was

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performed slowly, and the skin wound was sealed and treated with betadine solution. The experiment group of mice (20 animals) was given i.p. injection of 1 mM *N*-galloyl-4-alkyliden-β-lactam in 40 µl of PBS, at 9 am and 6 pm every day, excluded Sunday, starting 3 days before (10 mice) or the same day of (10 mice) instillation; the control group was left untreated.

All experiments were conform to the regulatory standards, and approved by the Medical School Committee of Padova.

Preparation of the tissues - Four weeks after intra-tracheal instillation, the mice were weighed, anesthetized, heparinized, and exsanguinated via the femoral artery. The heart and lungs were removed en bloc; the lungs were dissected away from the external vasculature and bronchi, and sectioned parasagittally, superior to inferior. Liver and kidneys were also explanted, and all the specimens were fixed in buffered 4% paraformaldehyde for morphological studies.

Histology - Fixed lung tissues were embedded in paraffin and stained with hematoxylin-eosin (H&E) and Heidenhain trichrome. The severity of inflammatory cell infiltration, the extension of interstitial fibrosis and the entity of alveolar cuboidalization was evaluated using a semiquantitative analysis (as lung parenchyma involved): score 0 = absent, score 1 = 1-30%, score 2 = >30 <60%, and score 3 = >60%. Heart, liver and kidney were also histologically processed to detect eventual toxic pathologic changes.

Determination of hydroxyproline - The hydroxyproline content of mouse lung was determined by standard methods (Moore et al., 2001), with slight modifications. After rinsing with PBS, the upper left lung lobe was de-fatted, dried, weighed, and hydrolyzed 22 h at 110°C in 6 N HCl. Aliquots were then assayed by adding chloramine T solution for 20 min, 3.15 M perchloric acid for 5 min, and Erlich's reagent at 60 °C for 20 min. Absorbance was measured at 561 nm, and the amount of hydroxyproline determined against a standard curve.

Statistics - Data are expressed as the means of three determinations for biochemical, and quadruplicates for migration and invasion assays (four PMN donors separately). Morphological examination was performed, analyzing at least two sections from each lung per animal and the data averaged both as mean score (MS) and mean percentage (MP), separately for the two

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experimental groups (with and without three-day pretreatment). Comparisons were conducted by one-way ANOVA, with significance set at $p < 0.05$.

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Results

Synthesis of the *N*-galloyl-4-alkyliden-β-lactam – The 4-alkyliden-azetidin-2-ones represent a relatively new class of β-lactam compounds: few examples were studied in the 1980s (Prasad et al., 1982; Greengrass and Hoople, 1981), with no further development. These compounds have been recently obtained using an original and well-established protocol starting from 4-acetoxy-azetidinones and diazoesters (Cainelli et al., 2003). We demonstrated that the unsaturated system on the alkyliden side chain activates these monocyclic β-lactams towards serine-proteases such as human LE (Prasad et al., 1982). *N*-galloyl-4-alkyliden-β-lactam was prepared with a two steps synthesis (Fig.1): $AlCl_3$-mediated addition of ethyl diazoacetate to 3-(2-*tert*-butyldimethylsilyloxyethyl)-4-acetoxy-azetidin-2-one, and subsequent *N*-acylation reaction with tri-*O*-benzyl-galloyl chloride (Cainelli et al., 2005; Prasad et al., 1982). ^{1}H and ^{13}C NMR analysis confirmed the chemical structure presented in Fig.1C for the target compound.

The oxidant property of the new compound was tested with the TEAC analysis, which registered an extremely low Trolox-equivalent value (17.3 μM in 6 min, from 2.12 mM *N*-galloyl-4-alkyliden-β-lactam in ethanol), indicating that the compound has no relevant anti-oxidant activity. The pH value of all buffers containing this compound as described in this section was always found unmodified.

Inhibition of proteolytic activities - When the three PMN's proteases (LE, cathepsin G, PR-3) were pre-incubated with *N*-galloyl-4-alkyliden-β-lactam, and then mixed with their specific synthetic substrates, a dose-dependent inhibition was registered, which maintained a constant slope throughout the 2 h of measurement. The 60 min plot shows that the IC_{50} for LE is quite low (below μM), while that of cathepsin G and PR-3 are over 100 μM (Fig.2A). The double-reciprocal plotting of the results obtained at different β-lactam concentrations for LE – sharing a common -1/Michaelis constant (Km) on the abscissa – reveals that this inhibition is non-competitive, with a Ki = 0.7 μM (Fig.2B).

The effect of the *N*-galloyl-4-alkyliden-β-lactam was also studied on the MMP-2 and MMP-9 activities contained in a culture medium conditioned by tumor cells (Garbisa et al., 1999). When the β-lactam was present (0-100 μM) in the buffer during the development of gelatin-zimography of the medium, the MMP-9-corresponding digestion bands were not

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inhibited, and those of MMP-2 were inhibited only by 25% at the highest concentration (not shown).

Effects on chemotaxis and chemoinvasion - The effect of the *N*-galloyl-4-alkyliden-β-lactam on PMN migration toward fMLP was measured by using a modified Boyden chamber assay. On gelatin-coated filters (chemotaxis), PMNs showed a 2-fold increase in migration to fMLP over un-stimulated controls; in this case, 15 min pre-treatment with *N*-galloyl-4-alkyliden-β-lactam – and its presence during the assay – did not affect PMN chemotaxis, within the tested range of concentrations (0-6 μM) (Fig.3). Controls with the β-lactam in the lower chamber excluded that the compound itself has chemotactic activity (not shown).

In contrast, on filters coated with reconstituted basement membrane, Matrigel (chemoinvasion), the invasive capacity of PMNs toward fMLP was restrained in a dose-dependent manner by *N*-galloyl-4-alkyliden-β-lactam (0 – 6.3 μM), with an $IC_{50} < 2$ μM (Fig.3). No evident effect on chemoinvasion was exerted by the precursor molecule (4-alkyliden-β-lactam, compound 2 in Fig.1A) at the same concentrations.

PMN's short-term viability (4 and 20 h) was not significantly affected (-3%) by the *N*-galloyl-4-alkyliden-β-lactam within the concentration range tested for chemoinvasion; only after 20 h incubation at 25 μM β-lactam (and in absence of FCS) a 15% decrease in viability was measured (not shown).

Effects on MMP-9 activation by LE - Usually, the medium conditioned 1-3 h by freshly isolated PMNs contains a prevalent gelatinolytic activity which is recognized as the zymogen form of MMP-9, and a limited proportion of this enzyme present in the activated form (varying from 9:1 to 8:2 ratio in different preparations). Preliminary data indicated that – within the range of concentrations used – the new β-lactam does not inhibit LE release from PMNs (data not shown); but, in order to boost the LE-triggered MMP-9 activation, aliquots of culture medium obtained from PMNs were incubated at 37 °C for 2 h with 5 mU of purified LE; in the presence of μM *N*-galloyl-4-alkyliden-β-lactam, the conversion of the zymogen to the activated form of MMP-9 was restrained in a dose-dependent manner, with >40 % decrease at 0.23 μM, and >80% at 6.3 μM, as verified by gelatin-zymography and densitometry (Fig.4).

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Effects on induced pulmonary fibrosis - Pulmonary fibrosis was induced in mice by intra-tracheal instillation of bleomycin, and the experiment group of mice was given *i.p.* injection of *N*-galloyl-4-alkyliden-β-lactam every day for four weeks, while the control group was left untreated (as described in details in the Methods).

At the end of the experiment, the weight of mice treated *i.p.* with the β-lactam **[**(+)β-lactam**]** showed no significant differences compared with controls [(-)bleomycin], and – from the start of the experiment (T_0) – registered a gain 2.36 times higher than that of the (–)β-lactam mice (from 17.0 g to 20.3 g and 18.4 g, respectively; $p < 0.01$) (Fig.5). No side effects were noted throughout the experiment. Intra-peritoneal administration of the compound twice daily for 31 days, starting 3 days before bleomycin treatment, registered some non-marginal effects even before animal sacrifice: all 10 (+)β-lactam mice appeared healthy during the experiment, while of the 12 (–)β-lactam controls 2 died after 8-10 days, and 6 presented signs of dyspnea, ataxia, and characteristic fur changes the second week after treatment. A similar effect was registered without 3-day pre-treatment (not shown).

The potential protective effect of the β-lactam on the bleomycin-induced pathology was thus studied by histological examination. The results obtained with and without three-day β-lactam pretreatment were very similar, and we now report only the former. Significantly less extensive inflammation was observed in (+)β-lactam animals than (–)β-lactam controls: mean score (MS) 0.9 *vs* 1.7 ($p=0.02$), and mean percentage (MP) of lung parenchyma involved 8% *vs* 35% ($p=0.01$) (Fig.5). Fibrosis was significantly less evident in treated animals than control group: (MS) 0.3 *vs* 0.9 ($p=0.02$) and (MP) 2% *vs* 11% ($p=0.04$). A significant difference was also observed for the alveolar changes (cuboidalization) in terms of MS (0.6 *vs* 1.6 and $p=0.03$), while not in terms of MP (2% *vs* 22%, $p=0.08$).

To quantitatively determine the extent of fibrosis, hydroxyproline content in the lung was measured as a surrogate for lung collagen deposition. While in untreated animals the value was 12.3 μg/lobe, in those treated with bleomycin the values measured four weeks after instillation were 15.8 and 14.2 μg/lobe with $p<0.01$, in (-)β-lactam and (+)β-lactam respectively.

No degenerative changes were observed in heart, liver and kidney tissues, thus excluding drug toxicity.

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Discussion

Here we describe the activity of a new *N*-galloyl-4-alkyliden-β-lactam exhibiting the most potent inhibition of LE so far reported for this group, and only one order of magnitude lower than the endogenous α1-PI; this primary effect leads to restrain MMP-9 activation, containment of PMN recruitment, and eventually marked down-grading of inflammation-triggered pulmonary fibrosis.

Chemistry and Biochemistry - The addition of a galloyl group – with *O*-benzyl protection of all three hydroxy groups – to the nitrogen atom of 4-alkyliden-β-lactam markedly increases the inhibition exerted on LE, in comparison to the precursor molecule: the LE activity is inhibited >95% by the new compound at 10 μM, but only 66% by 100 μM precursor-β-lactam (Cainelli et al., 2003a; Cainelli et al., 2005). While the new compound inhibits LE activity in a dose-dependent non-competitive manner, with Ki in the under-μM range (0.7 μM), the precursor showed a non-constant slope of inhibition, precluding calculation of Ki. As for the majority of the 4-alkylidene-azetidin-2-ones tested, the registered inhibition was maintained with a constant slope over the 2-h period of measurement, suggesting a stable effect at body temperature.

The new *N*-galloyl-4-alkyliden-β-lactam is thus the most potent LE inhibitor of its class, and the presence of the polyphenolic substituent in this β-lactam was a good choice for boosting this activity. This new compound is as good as EGCG, the most powerful exogenous inhibitor of LE so far described (IC_{50} = 0.4 μM) (Sartor et al., 2002b), and – like EGCG – is not as well active against Proteinase-3 and cathepsin G (IC_{50} > 100 μM), also released by PMNs. In contrast, the inhibition of MMP-2 and MMP-9, two gelatinases instrumental in cell invasion, is markedly lower (IC_{50} > 100 μM *vs* 10-30 μM) (Garbisa et al., 1999), widening the span between the efficacy on gelatinases and LE, and thus rendering the β-lactam even more specific for this potent proteolytic weapon. In addition, and in line with the effects obtained with other LE inhibitors (Clemente et al., 2001), the new β-lactam hinders the LE-triggered activation of the zymogen form of MMP-9, the major protease involved in PMN migration across basement membrane (BM) during extravasation (see also below).

We have previously shown that the galloyl group is essential for conferring to the catechins their anti-LE properties (Garbisa et al., 2000; Sartor et al., 2002b), and in the attempt to boost this character on β-lactams we thus designed a new series of compounds with a phenolic group added

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to the nitrogen atom of the β-lactam ring (Cainelli et al., 2005). The excellent anti-LE property exerted by the *N*-galloyl-4-alkyliden-β-lactam, in which the three hydroxyls are protected by benzyl residues, now confirms that while the galloyl framework is crucial for the inhibitory activity toward LE, the free hydroxyls are not. The latter were indeed shown to be mostly important in conferring anti-gelatinolytic properties (Sartor et al., 2002b).

As a preliminary approach, the possibility has been excluded that this anti-LE property may descend from anti-oxidant characteristics (well known – in contrast – for the catechin group, from which the galloyl residue has been here adopted). Some authors reported that the mechanism of LE inhibition by β-lactams involves a reaction with the nucleophilic serine-195 at the active site of elastase, which opens the β-lactam ring to form a stable acyl-enzyme intermediate (Knight et al., 1992); but recently other mechanisms have been defined (Taylor et al., 1999; Gerard et al., 2004). While the non-competitive inhibition registered for the new compound excludes the binding of this β-lactam to the LE active site, and should run off the former mechanism, no hints are yet available to suggest the latter or any other. In any case, the new β-lactam exerts an inhibition much superior than other natural and synthetic inhibitors of LE, in particular some standard class-specific serine-proteinase inhibitors such as PMSF and aprotinin (Sartor et al., 2002b), is only one order of magnitude less potent than the endogenous inhibitor α1-PI (Campbell et al., 2000), and exerts this property at non-cytotoxic concentration.

PMNs *in vitro* - Leukocyte extravasation and trafficking into the tissues is a crucial event in the host defense response, but over-activation and migration of PMNs to the site of inflammation – with their disruptive proteolytic load – can negatively contribute to inflammatory tissue injury, and lead to eventual pathological tissue alterations. We now show that PMN capacity of invading *in vitro* a reconstituted BM barrier (Matrigel) is markedly restrained by μM concentrations of the new β-lactam (IC_{50} < 2 μM). While these concentrations do not affect PMN's viability, our biochemical results suggest that a key role in this down-modulation is played (directly) by the inhibition of degradation of BM-components by LE activity, and (indirectly) the consequent impairment of LE-triggered activation of MMP-9.

Here it is worth mentioning that μM EGCG [green tea phytofactor which pivotal role of the galloyl moiety in LE inhibition was first demonstrated (Sartor et al., 2002b)] efficiently inhibits PMN chemotaxis and inflammatory recruitment (Donà et al., 2003). In contrast, the new compound – obtained by adding the galloyl moiety to the lactam ring – while restraining

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chemoinvasion, is completely un-effective on PMN chemotaxis: this suggests that it does not affect the cytoskeleton, while impairs mainly the proteolytic machinery. Preliminary data, indicating that it does not inhibit LE release from PMNs, seem also to exclude an effect on exocytosis processes.

The same dissected effects have been reported for PMNs treated with TIMP-1, the preferential MMP-9 inhibitor: also in this case, trans-BM migration was inhibited without affecting chemotaxis or degranulation (Delclaux et al., 1996). All together, these results strongly suggest that MMP-9 is a major factor on PMN migration across BM, and that LE is instrumental to this process by activating pro-MMP-9. This hypothesis is now reinforced by the clear dose-dependent inhibition of both chemoinvasion (Fig.3) and LE-triggered MMP-9 activation *in vitro* by □M *N*-galloyl-4-alkyliden-β-lactam (Fig.4).

Despite information on absorption, metabolism, and bioavailability of this new β-lactam were not yet available, we verified whether this effect, registered *in vitro*, is efficaciously translated *in vivo* in the restrain of inflammatory cell recruitment and consequent tissue damage (see below).

Pulmonary fibrosis - Chronic inflammation of the alveolar space is thought to mediate the development of pulmonary fibrosis, a disorder with an aggressive course (5-year survival of 50%) that is characterized by fibroblast proliferation and extra-cellular matrix remodeling, resulting in irreversible distortion of the lung's architecture (Selman et al., 2001). Intra-tracheal bleomycin challenge is a model of acute lymphocyte-dependent lung injury resulting in areas of patchy and chronic inflammation, with release of proteases, and production of cytokines, chemokines, and growth factors that mediate the eventual subpleural, peribronchiolar, and perivascular deposition of extracellular matrix and scar tissue formation that are characteristic of pulmonary fibrosis (Moore et al., 2001). Protection from induced pulmonary fibrosis in chemoattractant chemokine receptor knockout (CCR2 -/-) mice, in comparison with wild type, was shown to be independent from differential recruitment of inflammatory cells, and no statistical differences were registered in the PMN populations (Corbel et al., 2002).

Here, histological staining and semiquantitative analysis clearly showed that in animals given *i.p. N*-galloyl-4-alkyliden-β-lactam both the inflammatory-cell infiltration and the patchy fibrosis were significantly reduced (by 78% and 81% as mean percentage, respectively) (Fig.5), and quantitative determination of hydroxyproline (a marker of collagenous proteins) confirmed a

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significant reduction (-46%) of inflammation-triggered extracellular matrix collagen fiber deposition. A contributory factor to the anti-fibrotic effect might well be the inhibition exerted by *N*-galloyl-4-alkyliden-β-lactam on LE-triggered activation of MMP-9, the gelatinase involved in the damage to lung extracellular matrix (Egeblad and Werb, 2002; Lazo and Hoyt, 1990), and whose level significantly increases in induced pulmonary fibrosis concomitantly with collagen deposition (Selman et al., 2001), as above illustrated.

Cuboidalization, a histological marker of epithelial reactive changes, was also less evident in the treated animals: the lower epithelial damage parallels indeed the dramatically reduced inflammatory infiltration in the lungs of treated animals. Although the principal target and the mechanism of bleomycin-triggered lung injury are not well identified (van Acker et al., 1995), the possibility of a direct protective effect of this β-lactam on alveolar epithelial cells may not be excluded, and deserves further investigation.

Conclusions - To what extent the anti-fibrosis effect exerted by the *N*-galloyl-4-alkyliden-β-lactam is mainly the result of the restraint in the PMN default activity and turnover, and how much of the down-modulation of other cells (Cainelli et al., 2003b) remains to be determined. Certainly, this new β-lactam is a potent inhibitor of one of the most aggressive PMN protease (LE), and may be effective in preventive and therapeutic treatment of individuals exposed to risk of excessive or chronic inflammation.

Recurrent inflammation is considered a potential causative step of tumoral transformation in some types of tissue, and inflammatory infiltration of the primary mass is a common occurrence (Balckwill and Mantovani, 2001). Whether or not the inhibition exerted by the new compound on the LE-triggered activation of MMP-9 – which is instrumental also in cancer invasion (Lazo and Hoyt, 1990) – successfully hinders the metastatic aggressiveness of tumor cells is actually under investigation.

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a) unnumbered footnote to the title :

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b) reprint request to :

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1. **Authorship** IDA and LS contributed equally to the present work

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Figure Legends

Figure 1 : **A**) Scheme of synthesis of compounds **1** and **2** (4-alkyliden-β-lactam); **B**) structure of (-)epigallocatechin-3-gallate (EGCG) with encircled galloyl moiety; **C**) scheme of synthesis of compound **3**, here named *N*-galloyl-4-alkyliden-β-lactam.

Figure 2 : Inhibition of PMN's proteases by the *N*-galloyl-4-alkyliden-β-lactam. **A**) Leukocyte elastase (LE), Proteinase-3 (PR-3), and cathepsin G (cat.G) were pre-incubated 15 min with increasing concentration of the β-lactam, then the specific chromogenic substrates were added, and the mixture incubated 60 min at 37 °C. The absorbance (405 nm) of triplicate digestions was averaged (S.D. always < 10%) and expressed as percentage of controls. In the same conditions, the precursor molecule (2 in Fig.1A) at 100 μM inhibits only 66% of LE activity (Cainelli et al, 2003a). **B**) The double-reciprocal plot demonstrates non-competitive inhibition of LE by increasing concentration of compound (mean values of triplicate samples, with S.D. <10%). S = substrate.

Figure 3 : Chemoinvasion – but not chemotaxis – of PMNs is inhibited by *N*-galloyl-4-alkyliden-β-lactam. The modified Boyden chamber assay shows that: PMN chemotaxis through gelatin (open circles), toward fMLP in the lower chamber, is not restrained by the β-lactam present in the upper chamber; while a parallel experiment shows that PMN chemoinvasion through Matrigel (full circles) is restrained in a dose-dependent manner, with an IC_{50} = 1-2 μM. Examples of quadruplicate experiments (45 min A; 2 h B); average of quadruplicate ± SD.

Figure 4 : Inhibition of LE-triggered activation of pro-MMP-9 by *N*-galloyl-4-alkyliden-β-lactam. Gelatin zymography of equal volumes of MMP-9 gelatinase-containing culture medium, pre-incubated 2 h at 37 °C with 0.5 mU of commercial LE, and increasing concentrations of β-lactam. Numbers in white = percentage of inhibition, as from densitometry of the activated form of MMP-9.

Figure 5 : Daily *i.p. N*-galloyl-4-alkyliden-β-lactam protects mice from inflammation-triggered

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pulmonary fibrosis. Foci of inflammatory infiltration (arrows in A; H&E), cuboidalization of epithelial cells (arrows in B), and peribronchial and interstitial fibrosis (blue-green in C; Heidenhaim Trichrome) are clearly visible in example-lungs of bleomycin-treated mice, while reduced or normal parenchyma is seen in bleomycin-plus-β-lactam-treated animals after 4 wk (D and E: H&E; F: Heidenhaim Trichrome). All histologies are at 25x. Image analysis quantitation confirms the reduction of bleomycin-induced inflammation (histogram 1), cuboidalization (histogram 2), and fibrosis (histogram 3) in bleomycin-plus-β-lactam-treated mice. Eventually, the weight gain (from T_0) in bleomycin-plus-β-lactam-treated mice was significantly higher than in those treated with bleomycin alone (histogram 4).

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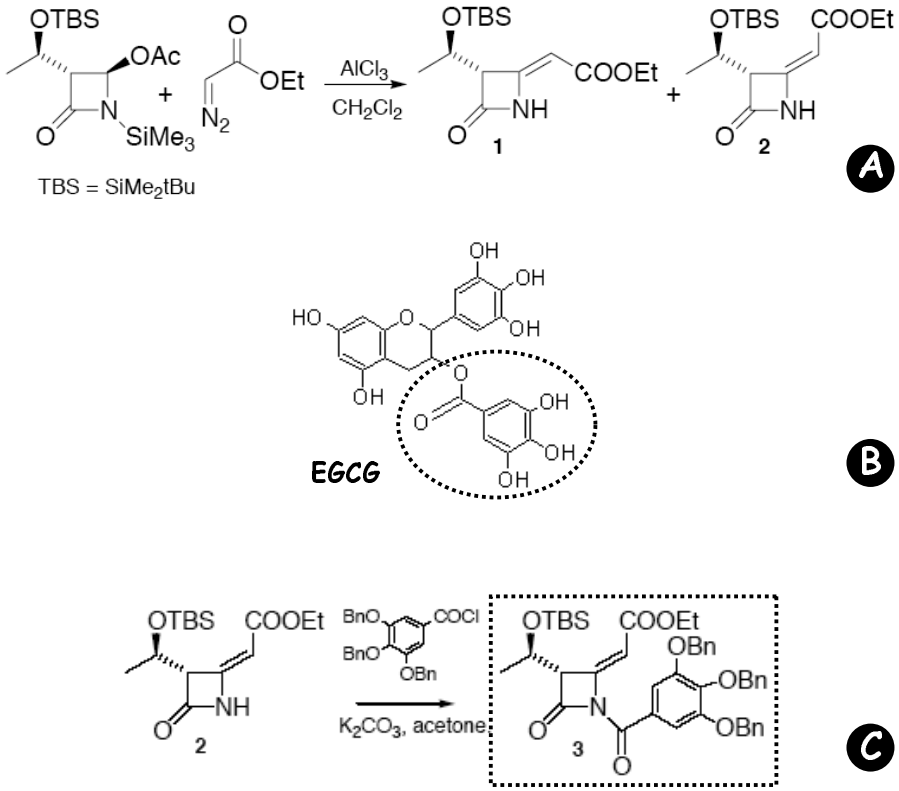

Fig. 1

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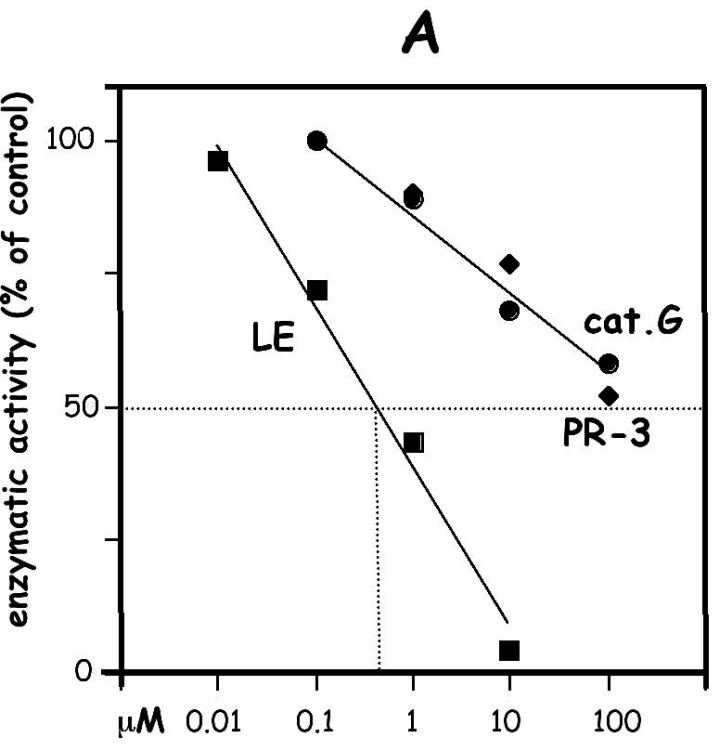

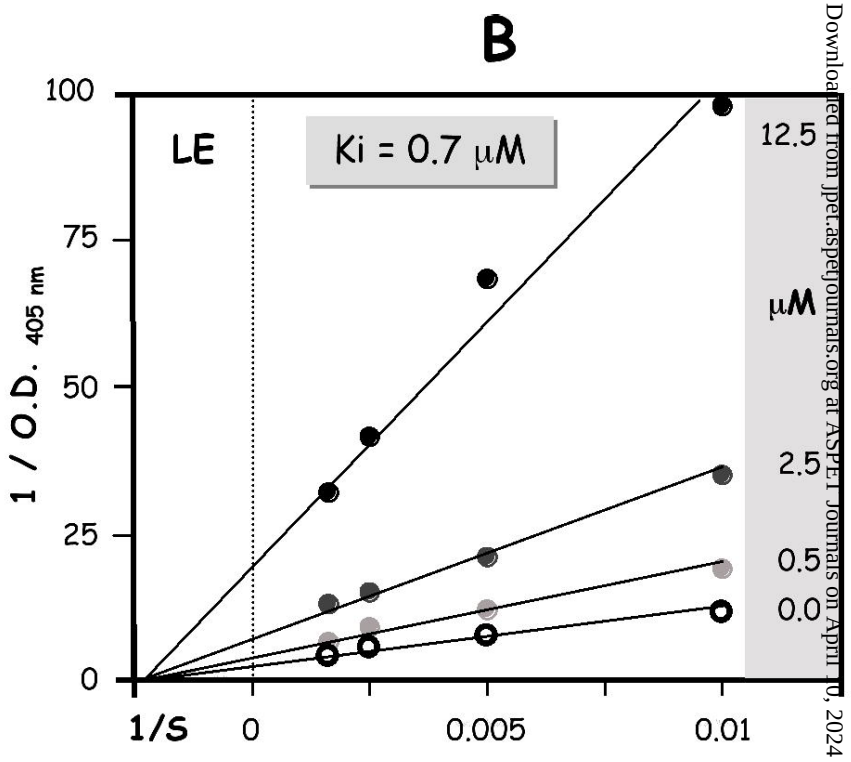

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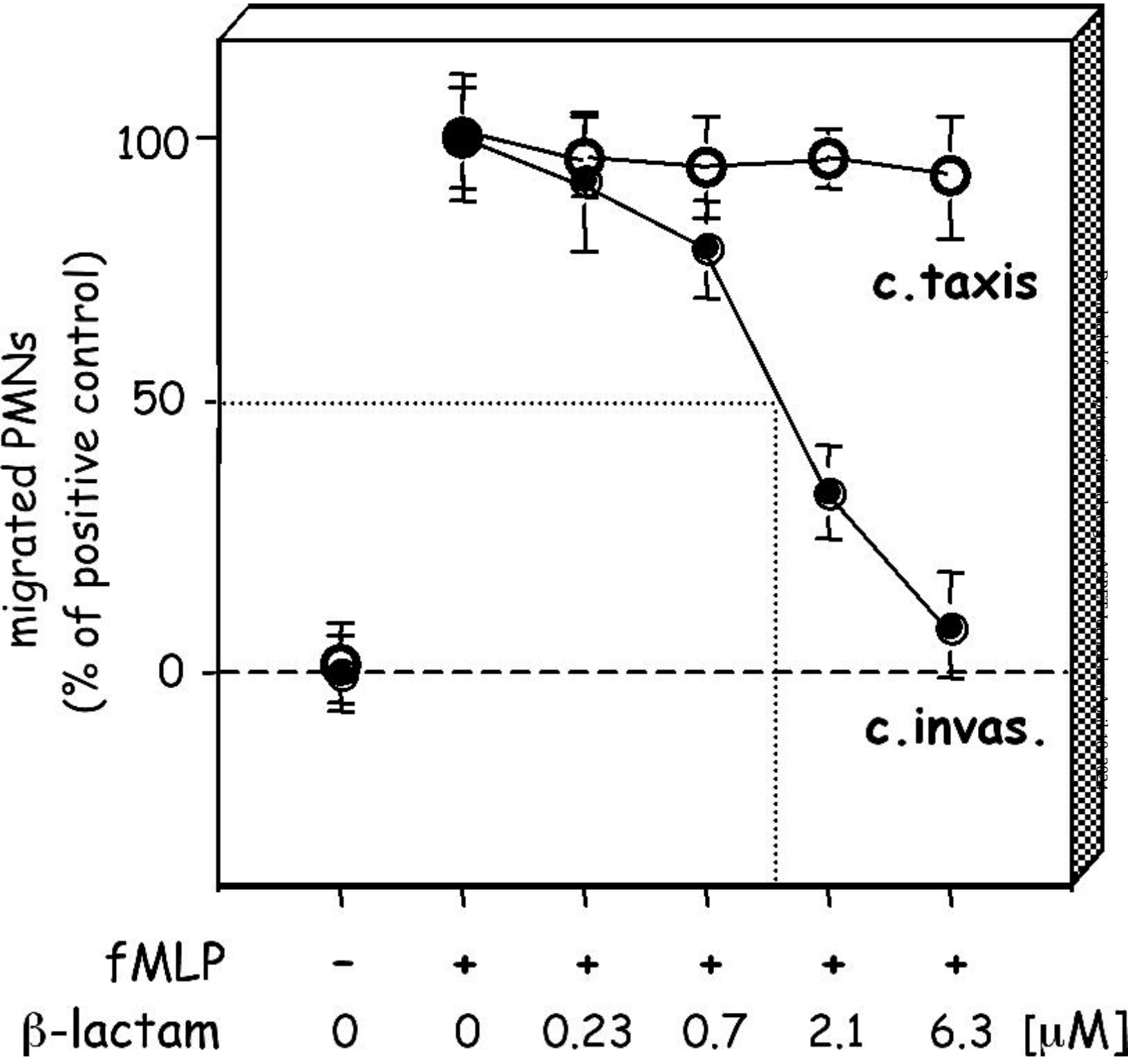

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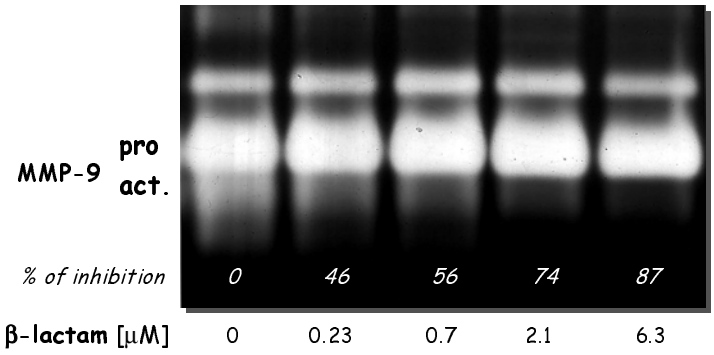

Fig. 4

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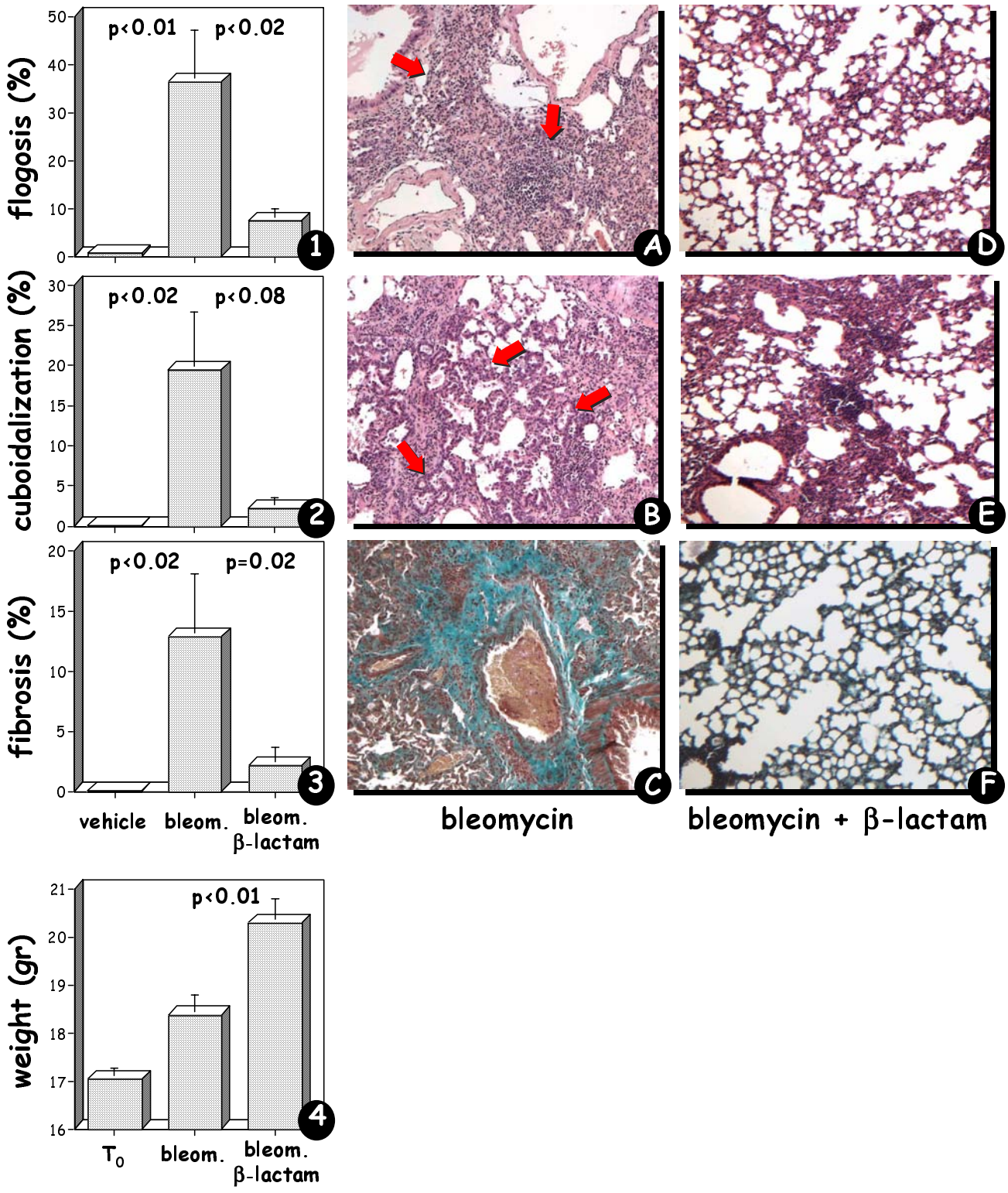

Fig. 5

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