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Inhibitors of poly (ADP-ribose) polymerase modulate signal transduction pathways and the development of bleomycin-induced lung injury.

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List of abbreviations: Poly(ADP-ribose) polymerase (PARP); nuclear factor-κB (NF-κB); spinal cord injury (SCI); reactive oxygen species (ROS); adenosine triphosphate (ATP); 3-aminobenzamide (3-AB); 1,5-dihydroxyisoquinoline (5-hydroxyisoquinolin-1(2H)-one) (1,5-DHIQ); 3,4-dihydro-5-(4-(1-piperidinyl)butoxyl)-1(2H)-isoquinolinone), (DPQ); 5-aminoisoquinolinone [5-aminoisoquinolin-1(2H)-one] (5-AIQ); Electrophoretic mobility-shift assay (EMSA); poly(ADP-ribose) (PAR); Myeloperoxidase (MPO);

ABSTRACT

Poly(ADP-ribose) polymerase, (PARP), a nuclear enzyme activated by strand breaks in DNA, plays an important role in the tissue injury associated with inflammation. The aim of our study was to evaluate the therapeutic efficacy of *in vivo* inhibition of PARP in an experimental model of lung injury caused by bleomycin administration. Mice subjected to intratracheal administration of bleomycin developed a significant lung injury and apoptosis (measured by ANNEXIN-V coloration). An increase of immunoreactivity to nitrotyrosine and PARP as well as a significant loss of body weight and mortality was observed in the lung of bleomycin-treated mice. Administration of the two PARP inhibitors, 3-aminobenzamide (3-AB) or 5-aminoisoquinolinone (5-AIQ) significantly reduced the (i) lost of body weight, (ii) mortality rate, (iii) infiltration of the lung with polymorphonuclear neutrophils (MPO activity), (iv) edema formation and (v) histological evidence of lung injury. Administration of 3-AB and 5-AIQ also markedly reduced the nitrotyrosine formation and PARP activation. These results demonstrate that treatment with PARP inhibitors reduces the development of inflammation and tissue injury events induced by bleomycin administration in the mice.

INTRODUCTION

Idiopathic pulmonary fibrosis is characterized by the excessive deposition of extracellular matrix in the lung interstitium (Krishna *et al.*,2001). The pathological features of inflammation and fibrosis are well appreciated but little is known about its etiology and pathogenesis.(Idiopathic pulmonary fibrosis, 2000). Various anti-inflammatory agents such as corticosteroids, (Mapel *et al.,* 1996) colchicine, (Douglas *et al.,* 1997) and cytotoxic agents such as azathioprine (Raghu *et al.,* 1991) and cyclophospamide (Johnson *et al.,* 1989) have been used alone or in combination to treat the disease. However, less than one-third of patients respond to treatment with corticosteroids and/or cytotoxic therapy.

In view of the poor outcomes and therapeutic options available in idiopathic pulmonary fibrosis and other fibrotic lung diseases, there is an urgent need for new insights into their pathobiology that can be translated into therapeutic alternatives (Peters-Golden *et al.,* 2002).

The production of reactive oxygen species (ROS) and peroxynitrite contribute to the tissue injury observed during lung fibrosis. ROS and peroxynitrite also cause DNA damage, which results in the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Szabó and Dawson 1998). Therefore, recently it been demonstrated that bleomycin administration induced PARP activation in the lung tissues (Genovese *et al.,* 2005 in press) PARP is a ubiquitous, chromatin-bound enzyme, which is abundantly present in the nuclei of numerous cell types (Szabó and Dawson 1998). Continuous or excessive activation of PARP produces a depletion of NAD^+ and subsequently leading to cellular dysfunction and ultimately, cell death (Chiarugi, 2002). Chemically distinct inhibitors of PARP activity such as benzamides [e.g., 3-aminobenzamide (3-AB), nicotinamide] and isoquinolinones can reduce the degree of inflammation and these investigations have provided the basis for potential clinical applications of PARP inhibitors (Southan and Szabò 2003).

Therefore, various studies have demonstrated that the chemically distinct PARP inhibitors GPI6150, PJ34 and 3-AB can attenuate PARP activation and provide beneficial actions *in vivo* during inflammation (Cuzzocrea *et al.,* 2002a;). However, in contrast isoquinolinone derivatives 3-AB, and nicotinamide are weak inhibitors of PARP activity that do not readily cross cell membranes (Szabó and Dawson, 1998). Furthermore, although the potency of recently developed PARP inhibitors has improved greatly, most lack good solubility in water, making it difficult to find a biocompatible vehicle for utilization *in vivo*. Thus, there is still a great need for the development of potent, water-soluble inhibitors of PARP activity. Much effort has been made to develop new PARP inhibitors with better potency, selectivity and water-solubility and there are now 13 chemical classes of PARP inhibitors (Southan and Szabò 2003). Twelve years ago, Suto et al (Suto *et al.,* 1991) used a cell-free preparation of PARP (purified 900-fold from calf thymus) to demonstrate that 5-aminoisoquinolinone [5-aminoisoquinolin-1(2*H*)-one] (5-AIQ) is a water-soluble inhibitor of PARP activity. As previously published reports of the synthesis of 5-AIQ reported problems of low yield and unreliability (Suto et al., 1991), McDonald and colleagues have recently developed a novel and more efficient method for the synthesis of 5-AIQ (McDonald et al., 2000).

This method leads to a higher yield of 5-AIQ than that previously reported (Wenkert et al., 1964). Suto et al (Suto et al., 1991) and Watson and colleagues (Watson et al., 1998) reported an IC_{50} of 240 nmol/L when 5-AIQ was evaluated in an in vitro cell free system consisting of PARP isolated from calf thymus, which is broadly comparable with other potent 5-substituted isoquinolinones. As 5-AIQ is an analogue of the nicotinamide moiety of NAD^+, it is conceivable that it may also inhibit other ADP-ribosyl transferases. We have previously examined the effect of 5-AIQ on the mono-ADP-ribosylating activity of diphtheria toxin and found that it had an IC_{50} of approximately 250 μmol/L, indicating a 1000-fold higher

selectivity for PARP (McDonald et al., 2000). In addition, when compared to the benzamides 3-AB and nicotinamide and the isoquinolinone 1,5-DHIQ, 5-AIQ is the most potent (water-soluble) inhibitor of PARP we have examined to date (Chatterjee et al., 1999; 2000). Furthermore, we have, also, previously demonstrated that 5-AIQ can reduce ischemia-/reperfusion injury of the heart, intestine and liver (Mota Filipe et al., 2002), and 5-AIQ has been shown to provide beneficial effects in rodent models of heart transplantation (Szabo et al., 2002), acute lung injury (Cuzzocrea et al., 2002a) and spinal cord injury (Genovese *et al.*, 2004). Consistent with these findings, the objective of the present study was to investigate the biological effects of pharmacological inhibition of PARP in a mouse model of lung fibrosis with two structurally unrelated inhibitors of PARP, 3-AB, as reference inhibitor and 5-AIQ as new synthetic inhibitor were tested.

MATERIALS AND METHODS

Animals

Adult male CD1 mice (25-30 g, Harlan Nossan, 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 purpose (D.M. 116192), as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Experimental groups

Mice were randomly allocated into the following groups: (i) *BLEO + vehicle group*. Mice were subjected to bleomycin-induced lung injury and received the administration of saline (*N*=30); (ii) *3-AB group*. Same as the *BLEO + vehicle group* but 3-AB at a dose of 10 mg/kg was administered (i.p.) bolus every 24h starting from day 1 (*N*=30); (iii) *5-AIQ group*. Same as the *BLEO + vehicle group* but 5-AIQ at a dose of 3 mg/kg was administered (i.p.) bolus every 24h starting from day 1 (*N*=30); (iv) *Sham + saline group.* . Sham-operated group in which identical surgical procedures to the BLEO group was performed, except that the saline was administered instead of bleomycin (*N*=30); (v) *Sham + 3-AB group.* Identical to *Sham + saline group* except for the administration of 3-AB (i.p.) bolus every 24h starting from day 1 (*N*=30); (vi) *Sham + 5AIQ group.* Identical to *Sham + saline group* except for the administration of 5AIQ (i.p.) bolus every 24h starting from day 1.

The dosage of PARP inhibitors regimen has been previously shown to exert anti-inflammatory effects. In particular, the dose of 5-AIQ have previously been reported by us to reduce the tissue injury caused by ischemia-reperfusion in the liver (dose-response curve study) (Mota-Filipe *et al.*, 2002) as well as lung injury (Cuzzocrea *et al.*, 2002a) and the dose

of 3-AB have previously been reported by us to reduce acute lung inflammation (dose-response curve study) (Cuzzocrea *et al.*, 1998).

Induction of lung injury by bleomycin

Mice received a single intratracheal instillation of saline (0.9%) or saline containing bleomycin sulphate (1 mg/kg body weight) in a volume of 50 µl and were killed after 15 days by pentobarbitone overdose.

Measurement of fluid content in lung

The wet lung weight was measured after careful excision of extraneous tissues. The lung was exposed for 48 h at 180°C and the dry weight was measured. Water content was calculated by subtracting dry weight from wet weight.

Histological examination

Lung biopsies were taken 15 days after injection of bleomycin. Lung biopsies were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). After embedding in paraffin, the sections were prepared and stained by trichrome stain. All sections were studied using light microscopy (Dialux 22 Leitz). The severity of fibrosis was semi quantitatively assessed according to the method proposed by Ashcroft and co-workers (Ashcroft *et al.*, 1988). Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining section randomly chosen fields per sample at a magnification of x100. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung

structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of fields.

Immunohistochemical localization of nitrotyrosine and PARP

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or ROS, was determined by immunohistochemistry as previously described (Cuzzocrea *et al.*, 2003). At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-nitrotyrosine polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-ribose) goat polyclonal antibody (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). In order to confirm that the immunoreactions for the nitrotyrosine were specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PAR some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane *et al.,* 1985). At the specified time following injection of bleomycin, lung tissues were obtained and weighed, each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x g at 4° C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37° C and was expressed in milliunits per g of wet tissue.

Measurement of cytokines

Portions of lung, collected at 15 days after bleomycin administration, were homogenized as previously described (Diaz-Granados *et al.,* 2000) in PBS containing 2 mmol/L of phenyl-methyl sulfonyl fluoride (Sigma Chemical Co.) and tissue levels of TNFα and IL-1β were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer instructions. All cytokines determinations were performed in duplicate serial dilutions.

Annexin -V- evaluation.

The binding of annexin V-fluorescein isothiocyanate (Ann-V) to externalized phosphatidylserine was used is a measurement of apoptotic in lung tissue section with an Ann-V-propidium iodide (PI) apoptosis detection kit (Santa Cruz, DBA Milan Italy) according to the manufacturer's instructions. Briefly, normal viable cells in culture will stain

negative for Annexin V FITC and negative for PI. Cells that are induced to undergo apoptosis will stain positive for Annexin V FITC and negative for PI as early as 1 hour after stimulation (Schutte *et al.,* 1998). Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. Both cells in later stages of apoptosis and necrotic cells will stain positive for Annexin V FITC and PI. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a LSM 510 Zeiss laser confocal microscope equipped with a 4OX oil objective.

Preparation of whole extracts

All the extraction procedures were performed on ice using ice-cold reagents. Tissues from each mouse were suspended in 6 ml of a high-salt extraction buffer (20 mM pH 7.9 HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and homogenized at the highest setting for 2 min in a Polytron PT 3000 tissue homogenizer. The homogenates were chilled on ice for 15 min and then vigorously shaken for a few minutes in the presence of 20 μl of 10 % Nonidet P-40. After centrifugation at 13,000 x g at 4°C for 5 min, the protein concentration in the supernatant was determined by the Bio-Rad (Bio-Rad) protein assay kit and then it was aliquoted and stored at –80°C.

Electrophoretic mobility-shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5'-GAT CGA GGG GAC TTT CCC TAG-3') were end labeled with γ-[^{32}P]ATP (ICN Biomedicals). Aliquots of whole extracts collected 15 days after bleomycin administration (20 μg of protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides (2.5 - 5.0 x

10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM ethylenediaminotetraacetic acid, 1mM DL-dithiothreitol, 1 mg/ml bovine serum albumin, 10 % glycerol. The specificity of the DNA/protein binding was determined for NF-κB by competition reaction in which a 50 fold molar excess of unlabeled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. Protein-nucleic acid complexes were resolved by electrophoresis on 4 % nondenaturing polyacrylamide gel in 0.5 % Tris borate ethylenediaminotetraacetic acid buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at –80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM). The time of 15 days after bleomicyn administration was chosen in agreement with other studies (Ortiz *et al.,* 2002)

Western blot analysis for IκB-α

The levels of IκB-α were quantified in whole extracts 14 days after bleomycin administration, by immunoprecipitation followed by Western blot analysis according to the manufacturers instructions (Celbio, Milan, Italy).

Briefly, proteins were then transferred onto nitrocellulose membranes, according to the manufacturer's instructions. Briefly, the membranes were saturated by incubation at 4°C overnight with 10 % (w/v) non-fat dry milk in PBS and then incubated with anti-IκB-α (1:1000) for 1 h at room temperature. Membranes were washed three times with 1% (w/v) Triton X-100 in PBS and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (1:1000). The immune complexes were visualized using the ECL chemiluminescence method (Amersham, Buckinghamshire, U.K.). Subsequently, the relative expressions of the proteins were quantified by densitometric scanning of the X-ray films with

GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) 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 compared with Student's unpaired t-test. A P-value of less than 0.05 was considered significant.

RESULTS

Effect of PARP inhibition on the degree of bleomycin-induced lung injury

Histological examination of lung sections revealed significant tissue damage (Fig. 1AD). Thus, when compared to lung sections taken from saline-treated animals (data not shown), histological examination of lung sections of mice treated with bleomycin were characterized by extensive inflammatory infiltration by neutrophils, lymphocyte and plasma cells extending through the lung epithelial, fibrosis and granulomas in perivascular region (Fig. 1AD). Administration of 3-AB or 5-AIQ significantly prevented lung inflammation induced by bleomycin administration (Fig. 1BC respectively; see Fig 1D for histological score). Furthermore, the injection of bleomycin elicited an inflammatory response characterized by the accumulation of water in lung as an indicator of fluid content, (Fig. 2) and neutrophils infiltration in the lung tissues (Fig. 3). The treatment with the two PARP inhibitors significantly reduced the fluid content and the neuthrophil infiltration (Figs. 2, 3).

Effects of PARP inhibition on changes of body weight and survival rate

In vehicle-treated mice, the severe lung injury caused by bleomycin administration was associated with a significant loss in body weight (Fig. 4). The treatment with the two PARP inhibitors 5-AIQ or 3-AB significantly reduced the loss in body weight (Fig. 4). The survival of animals was monitored for 15 days. Bleomycin-treated mice, which had received vehicle, developed severe lung injury and 50% of these animals died within 15 days after bleomycin administration (Fig 5). In contrast, none of the mice, which had been treated with the two PARP inhibitors died (Fig. 5).

Effect of PARP inhibition on the production TNF-α and IL-1β after bleomycin administration

To test whether PARP activation may modulate the inflammatory process through the regulation of the secretion of others cytokines, we analyzed the lung levels of pro-inflammatory cytokines TNF-α and IL-1β at 15 days after bleomycin administration. A substantial increase of TNF-α and IL-1β formation was found in lung samples collected from bleomycin-treated mice (Fig. 6). Lung levels of TNF-α and IL-1β were significantly reduced in bleomycin-treated mice which have been treated with the two PARP inhibitors (Fig. 6).

Effects of PARP inhibition on bleomycin-induced nitrotyrosine formation and PARP activation

To determine the localization of *"peroxynitrite formation"* and/or other nitrogen derivatives produced during colitis, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the lung. Immunohistochemical analysis of lung sections obtained from mice treated with bleomycin revealed a positive staining for nitrotyrosine manly localized in nuclei of inflammatory cells (Fig. 7A,9). In contrast, no positive staining for nitrotyrosine was found in the lungs of bleomycin-treated mice, which had been treated with 3-AB (Fig. 7B,9) or with 5-AIQ (Fig. 7C,9). Immunohistochemical analysis of lung sections obtained from mice treated with bleomycin also revealed a positive staining for PAR manly localized in plasma cell and lymphocytes (Fig. 8A,9). In contrast, no staining for PAR was found in the lungs of bleomycin-treated mice treated with 3-AB (Fig. 8B,9) or with 5-AIQ (Fig. 8C,9). There was no staining for either nitrotyrosine or PAR in lungs obtained from the sham group of mice (data not shown).

Effect of PARP inhibition on apoptosis in lung tissue after bleomycin administration

To test whether the tissue damage was associated with cell death by apoptosis, we measured Annexin V staining in the lung from bleomycin-treated mice at 15 days after bleomycin administration. Almost no apoptotic cells were detectable in the lung tissue of sham-treated mice (data not shown). At 15 days after bleomycin administration, lung tissues obtained from vehicle-treated mice demonstrated a marked appearance of positive staining to propidium iodide (Fig 10A) index of cells in the late stage of apoptosis as well as some positive stain positive for Annexin V FITC index of cells that are induced to undergo apoptosis (Fig. 10B). On the contrary, lung tissues section from bleomycin-treated mice, which had been treated with 3-AB (Fig. 10D) or with 5-AIQ (Fig. 10G) demonstrate significantly less presence of cells in the later stages of apoptosis (positive to propidium iodide) and almost no positive staining necrotic for Annexin V FITC was observed (Fig. 10E,H respectively).

Effect of PARP inhibitors on IκB-α degradation and NF-κB translocation

To investigate the cellular mechanisms by which treatment with 3-AB or 5-AIQ may attenuate bleomycin-induced lung injury, we evaluated IκB-α degradation and NF-κB translocation, one of the major transcription factors involved in the signal transduction of inflammation (Genovese *et al* 2004).

The appearance of IκB-α in homogenates of lung tissues was investigated by immunoblot analysis at 15 days after bleomycin administration. A basal level of IκB-α was detectable in the homogenated lung tissues from sham-treated mice (Figure 11). IκB-α levels were substantially reduced in the lung tissues from bleomycin-treated mice (Figure 11). PARP inhibition prevented such bleomycin-mediated IκB-α degradation and the IκB-α band remained unchanged 15 days after bleomycin administration in both the 3-AB and 5-AIQ treated mice (Figure 11). To detect NF-κB/DNA binding activity, whole extracts from lung

tissue of each mouse was analyzed by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from tissues of sham-treated mice (Figure 12). The DNA binding activity significantly increased in whole extracts obtained from lung tissues of vehicle-treated mice 15 days after bleomycin administration (Figure 12). Treatment of mice with 3-AB or with 5-AIQ caused a significant inhibition of bleomycin-induced NF-κB/DNA binding activity as revealed by specific EMSA (Figure 12).

The specificity of NF-κB/DNA binding complex was demonstrated by the complete displacement of NF-κB/DNA binding in the presence of a 50 fold molar excess of unlabeled NF-κB probe (W.T. 50x) in the competition reaction. In contrast a 50 fold molar excess of unlabeled mutated NF-κB probe (Mut. 50x) or Sp-1 oligonucleotide (Sp-1 50x) had no effect on this DNA-binding activity (data not shown).

DISCUSSION

The main findings of the current study are that treatment with 3-AB or 5-AIQ, two structurally unrelated PARP inhibitors attenuates: (i) the degree of PARP activation in the injured lung tissues, (ii) the infiltration of the injured lung with neutrophils, (iii) cell apoptosis, (iv) pro-inflammatory cytokines and (v) lung tissue damage. These protective effects were associated with inhibition of DNA binding of the transcription factor NF-κB in the inflamed lung. Thus, we propose that the anti-inflammatory activity of PARP inhibitors may be mediated, at least in part, by inhibition of the transcription of certain pro-inflammatory mediators, which are regulated by NF-κB. All of these findings support the view that PARP plays a detrimental role in the development and persistence inflammation associated with lung fibrosis in the mouse.

The pathological features of inflammation and fibrosis are well appreciated but little is known about its etiology and pathogenesis .(Idiopathic pulmonary fibrosis, 2000).

Bleomycin induction of lung injury in mice is a well established model of interstitial lung disease, resulting in pulmonary fibrosis (Smith *et al.,* 1996). Endotracheal instillation of bleomycin in mice is followed by upregulated expression of lung cytokines, development of lung inflammation, and accumulation of collagen in the lung (Smith *et al.,* 1996).

Earlier reports (Goodman *et al.,* 1998) point out that the pathogenesis of BLM-induced fibrosis, at least in part, is mediated through the generation of reactive oxygen species (ROS) which cause the peroxidation of membrane lipids and DNA damage. If that perspective is true, then antioxidant therapy may prevent the lung fibrosis caused by BLM and may prevent other diseases related with interstitial pulmonary fibrosis. Because BLM administration results in increased lipid peroxidation (LPO) and alters activities of antioxidant enzymes in bronchoalveolar lavage fluids (BALFs) and lung tissue (Karam *et al.,* 1998), in previous

studies (Venkatesan *et al.,* 1997) some natural or synthetic antioxidants have been used to protect against BLM oxidative lung toxicity both in vivo and also in vitro.

Recent studies have also suggested a contributory role for oxidants in gene induction. NF-κB is a pleiotropic transcription factor activated by low levels of ROS and inhibited by antioxidants (Cuzzocrea *et al.,* 2004). Consensus binding sequences for NF-κB have been identified in the promoter regions of several genes implicated in the pathogenesis of acute and chronic inflammation (Bowie and O'neill, 2000). In our experimental model of lung fibrosis , in agreement with previous reports (Ortiz et al., 2002), we found that DNA binding activity of NF-κB is increased at 15 days after bleomycin administration. Therefore, NF-κB DNA binding activity is associated with a significant IκB-α degradation in the lung tissues after bleomycin administration.

Thus, our data support the well-established hypothesis that NF-κB may represent an important therapeutic target in the treatment of inflammation (Bethea et al 1998). In this regard, we have recently demonstrated that pyrrolidine dithiocarbamate (PDTC), an antioxidant reported to be a potent inhibitor of NF-κB *in vitro*, significantly reduced acute lung inflammation by acting as an NF-κB inhibitor (Cuzzocrea *et al.,* 2002). Our present data show that the amelioration of lung damage by pharmacological inhibition of PARP was associated with inhibition of NF-κB activation. Although it is difficult to establish the definitive mechanism by which the PARP inhibitors reduce the DNA binding of these nuclear factors in *in vivo* experiments, our data support the possibility that PARP may be an important modulator of transcription during inflammation.

Moreover, in this study, the transient loss of IkB-α, which occurs in injured lung tissues from bleomycin-treated was prevented by PARP inhibitor treatment which correlated well with the inhibition of NF-κB activation suggesting that PARP inhibitors may also inhibit NF-κB activation via stabilization of IκB-α.

Our results are in agreement with other reports which have clearly demonstrated a role of poly (ADP-ribosyl)ation in signal transduction (Zingarelli *et al.,* 2003). Recently, it has been demonstrated that PARP-deficient cells are defective in NF-κB-dependent transcriptional activation (Oliver *et al.,* 1999). Similarly, pharmacological inhibitors of PARP abolish mRNA expression of iNOS, interleukin-6 and TNF-α *in vitro* cultured cells (Hauschildt *et al.,* 1997). Furthermore, recently Zingarelli and colleagues have clearly demonstrated that NF-κB DNA binding is completely abolished in heart from PARP-deficient mice subjected to ischemia and reperfusion (Zingarelli *et al.,* 2003), as well as, in the colon from rats subjected to experimental colitis treated with two different PARP inhibitors (Zingarelli *et al.,* 2003). The specific mechanism of PARP activation in regulating transcription needs future studies. Changes in cellular energetic after PARP activation may interfere with calcium sequestration and biosynthetic processes. Poly (ADP-ribosyl)ation may lead to the relaxation of chromatin with the consequence that genes become more accessible to RNA-polymerase (De Murcia *et al.,* 1988).

Numerous binding sequences of NF-κB on various genes with important immunologic functions characterize this transcription factor as a pluripotent factor in the inflammatory response (Xie *et al.,* 1994). Furthermore, the activation of NF-κB is a common end-point of various signal transduction pathways, including the activation of phosphatidylcholine-specific phospholipase C, protein kinase C, protein tyrosine kinases, and mitogen activated protein kinases and other signaling factors (Novogrodsky *et al.,* 1994). Binding of NF-κB to the respective binding sequence on genomic DNA encoding for different pro-inflammatory genes such us TNFα and IL-1β, results in a rapid and effective transcription of these genes (Xie et al., 1994). There is good evidence that TNFα and IL-1β help to propagate the extension of a local or systemic inflammatory process (Wooley *et al.,* 1993). Therefore, recently it has been demonstrated that TNFα plays a fundamental role in the pathogenesis of

bleomycin-induced pulmonary fibrosis (Ortiz *et al.,* 1998). We have previously reported that 5-AIQ inhibits TNF-α, and IL-1β production in an experimental model of lung inflammation induced by zymosan activated plasma (Cuzzocrea et al., 2002a). Therefore, the inhibition of the production of TNF-α and IL-1β by the two PARP inhibitors 5-AIQ and 3-AB described in the present study is most likely attributed to the inhibitory effect the activation of NF-κB. Another potential mechanism by which PARP inhibition improved lung injury in our experimental model of lung fibrosis is the reduction of neutrophil recruitment into the site of inflammation. Accumulation and activation of inflammatory cells are some of the initial events of tissue injury and are regulated at the transcriptional level. For example, expression of adhesion molecules, such as P-selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1), is regulated by genes responsive to NF-κB.

Therefore, we may hypothesize, as previously demonstrated (Cuzzocrea et al., 2002a) that pharmacological inhibition of PARP may also inhibit recruitment of inflammatory cells at the transcription level. The discovery of the concept that PARP regulates neutrophil trafficking may provide new insights in the interpretation of recent reports demonstrating the protective effect of PARP inhibition in experimental models of shock, ischemia-reperfusion injury and inflammation. For instance, there is good evidence that PARP activity (including 3-AB and 5-AIQ used in this study) reduces the upregulation of adhesion molecules (e.g. P-selectin and ICAM-1) in regional myocardial ischemia and reperfusion of the heart (Zingarelli *et al.,* 1997), the gut (Di Paola *et al.,* 2004), the kidney (Chatterjee *et al.,* 1999), as well as in the inflamed lung (Cuzzocrea *et al.,* 2002a). Therefore, recently it has been point out that the ability of PARP inhibitors in reducing infiltration of activated and PAR+ PMNs/monocytes into damaged tissues, may be independent of PARP activity. Consistent with this is the study by Scott and colleagues showing that peroxynitrite-induced oligodendrocyte cell death is PARP independent.

Extravasated polymorphonuclear leukocytes in the inflammatory sites become activated and produce a variety of inflammatory mediators such as growth factors, chemokines and cytokines, complement components, proteases, NO, reactive oxygen metabolites and peroxynitrite, which are important mediators of tissue injury (Cuzzocrea 2001). Prevention of neutrophil-dependent inflammatory pathways is likely to contribute to the improved histological status after inhibition of PARP. Thus, as previously indicated (Mazzon *et al.*, 2002), we propose the following positive feedback cycle in: early reactive oxygen species production >> PARP-related endothelial injury >> polymorphonuclear leukocyte infiltration >> more reactive oxygen species production. Inhibition of PARP would intercept this cycle at the level of endothelial injury. This model would explain the reduction of nitrotyrosine staining in lung tissues from bleomycin-treated mice which have been treated with the two PARP inhibitors: reduced neutrophil infiltration leads to reduced reactive oxygen species. Recent studies have demonstrated the induction of apoptosis in different cell line in response to ROS, peroxynitrite and nitric oxide (Leist et al., 1997).

It was reported that, in the lungs after bleomycin administration, apoptosis of bronchial and alveolar epithelial cells is observed (Kuwano *et al.,* 1999). Moreover, it was also reported that apoptosis of pneumocytes induced by agonistic antibody against Fas results in lung fibrosis (Hagimoto *et al.,* 1997). In the present investigation, FR-167653 administration markedly suppressed apoptosis of the lung cells. The role of p38 MAPK on apoptosis of various cell types is controversial, but it is possible that the suppression of apoptosis by FR-167653 is at least explained by inhibiting death signals transduced by Fas ligand and TNF-α whose expressions were enhanced by p38 MAPK (Zhang *et al.,* 2000). We demonstrate here that PARP inhibitors inhibit apoptotic cell death in lung tissues tissue from bleomycin-treated mice (as determined by Annexin V coloration). Thus, our *in vivo* findings support the view that inhibition of PARP directly protects cells by preventing the activation of the apoptosis

pathway. In conclusion, our results indicate that PARP inhibitors have strong anti-inflammatory properties resulting in reduced: 1) PMN infiltration, 2) activation of NF-κB, and 3) the degree of tissue injury.

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REFERENCES

Acta Vet Hung. 1991;39(3-4):215-24.

Ashcroft T, Simpson JM, and Timbrell V. (1988) Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* **41:**467-470

Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD, and Yezierski RP. (1998) Traumatic spinal cord injury induces nuclear factor-kappaB activation. *J Neurosci.* **18**:3251-60.

Bowie A, and O'neill LA. (2000). Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem. Pharmacol* **59**: 13-23.

Chatterjee PK, Cuzzocrea S, and Thiemermann C. (1999) Inhibitors of poly (ADP-ribose) synthetase protect rat proximal tubular cells against oxidant stress. *Kidney Int.* **56**:973-84.

Chatterjee PK, Zacharowski K, Cuzzocrea S, Otto, M and Thiemermann C (2000) Inhibitors of poly (ADP-ribose) synthetase reduce renal ischemia-reperfusion injury in the anesthetized rat *in vivo*. *FASEB J* **14**: 641-651.

Chiarugi A (2002) Poly(ADP-ribose) polymerase: Killer or conspirator? The 'suicide hypothesis' revisited. *Trends Pharmacol Sci* **23**: 22-129.

Cuzzocrea S, Zingarelli B, Gilard E, Hake P, Salzman AL, Szabó C. (1998). Protective effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthase in carrageenan-induced models of local inflammation. *Eur J Pharmacol* **342**, 67-76.

Cuzzocrea S, Chatterjee PK, Mazzon E, Dugo L, Serraino I, Britti D, Mazzullo G, Caputi AP, and Thiemermann C. (2002) Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. *Br J Pharmacol*. **135**:496-510.

Cuzzocrea S, Ianaro A, Wayman NS, Mazzon E, Pisano B, Dugo L, Serraino I, Di Paola R, Chatterjee PK, Di Rosa M, Caputi AP, and Thiemermann C. (2003) The cyclopentenone prostaglandin 15-deoxy-delta-(12,14)-PGJ2 attenuates the development of colon injury caused by dinitrobenzene sulphonic acid in the rat. *Br J Pharmacol* **138**:678-88.

Cuzzocrea S, McDonald MC, Mazzon E, Dugo L, Serraino I, Threadgill M, Caputi AP, and Thiemermann C. (2002a) Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase, in a rodent model of lung injury. *Biochem Pharmacol.* **63**:293-304.

Cuzzocrea S, Pisano B, Dugo L, Ianaro A, Ndengele M, and Salvemini D. (2004) Superoxide-related signaling cascade mediates nuclear factor-kappaB activation in acute inflammation. *Antioxid Redox Signal.* **6:**699-704.

Cuzzocrea S, Riley DP, Caputi AP, and Salvemini D. (2001) Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol Rev.* **53**:135-59. Review.

de Murcia G, Huletsky A, and Poirier GG (1988) Modulation of chromatin structure by poly(ADP-ribosyl)ation. *Biochem Cell Biol* **66**:626-35.

Di Paola R, Genovese T, Caputi AP, Threadgill M, Thiemermann C, and Cuzzocrea S. (2004) Beneficial effects of 5-aminoisoquinolinone, a novel, potent, water-soluble, inhibitor of poly (ADP-ribose) polymerase, in a rat model of splanchnic artery occlusion and reperfusion. *Eur J Pharmacol*. **492**:203-10.

Diaz-Granados N, Howe K, Lu J, and McKay DM. (2000) Dextran sulfate sodium-induced colonic histopathology, but not altered epithelial ion transport, is reduced by inhibition of phosphodiesterase activity. *Am J Pathol* **156**:2169-77.

Douglas WW, Ryu JH, Bjoraker JA, Schroeder DR, Myers JL, Tazelaar HD, Swensen SJ, Scanlon PD, Peters SG, and DeRemee RA. (1997) Colchicine versus prednisone as treatment of usual interstitial pneumonia. *Mayo Clin Proc.* **72**:201-9.

Genovese T, Cuzzocrea.S, Di Paola R, Mastruzzo C, Catalano P, Dugo L, Sortino M, Crimi N, Caputi AP, Thiemermann C and Vancheri C. (2005) Effect of rosiglitazone and 15-deoxy-$D^{12,14}$-prostaglandin J_2 on bleomycin-induced lung injury. *European Respiratory Journal* In press

Genovese T, Mazzon E, Muia C, Patel NS, Threadgill MD, Bramanti P, De Sarro A, Thiemermann C, and Cuzzocrea S. (2004) Inhibitors of poly (ADP-ribose) polymerase

modulate signal transduction pathways and secondary damage in experimental spinal cord trauma. *J Pharmacol Exp Ther*.

Goodman MT, Hernandez B, Wilkens LR, Lee J, Le Marchand L, Liu LQ, Franke AA, Kucuk O, and Hsu TC. (1998) Effects of beta-carotene and alpha-tocopherol on bleomycin-induced chromosomal damage. *Cancer Epidemiol Biomarkers Prev*. **7:**113-7.

Hagimoto, N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, and Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. *Am J Respir Cell Mol Biol* **17**: 272-278, 1997

Hauschildt S, Scheipers P, Bessler W, Schwarz K, Ullmer A, Flad HD, and Heine H (1997) Role of ADP-ribosylation in activated monocytes/macrophages. *Adv Exp Med Biol* **419**:249-52

Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement. *Am J Respir Crit Care Med* 2000; **161**:646-64.

Johnson MA, Kwan S, Snell NJ, Nunn AJ, Darbyshire JH, and Turner-Warwick M. (1989) Randomised controlled trial comparing prednisolone alone with cyclophosphamide and low dose prednisolone in combination in cryptogenic fibrosing alveolitis. *Thorax*.**44**:280-8.

Jones CB, McIntosh J, Huang H, Graytock A, and Hoyt DG. (2001) Regulation of bleomycin-induced DNA breakage and chromatin structure in lung endothelial cells by integrins and poly(ADP-ribose) polymerase. *Mol Pharmacol*. **59**:69-75.

Karam H, Hurbain-Kosmath I, and Housset B. (1998) Antioxidant activity in alveolar epithelial type 2 cells of rats during the development of bleomycin injury. *Cell Biol Toxicol.* **14**:13-22.

Krishna G, Liu K, Shigemitsu H, Gao M, Raffin TA, and Rosen GD. (2001) PG490-88, a derivative of triptolide, blocks bleomycin-induced lung fibrosis. *Am J Pathol*.**158**:997-1004

Kuwano, K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, Suda T, Kunitake R, Maeyama T, Miyazaki H, and Hara N. (1999) Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. *J Clin Invest* **104**: 13-19

Leist M, Single B, Kunstle G, Volbracht C, Hentze H, and Nicotera P. (1997) Apoptosis in the absence of poly-(ADP-ribose) polymerase. *Biochem Biophys Res Commun*. **233**:518-22.

Mapel DW, Samet JM, and Coultas DB. (1996) Corticosteroids and the treatment of idiopathic pulmonary fibrosis. Past, present, and future. *Chest*.**110**:1058-67. Review.

Mazzon E, Dugo L, De SA, Li JH, Caputi AP, Zhang J, and Cuzzocrea S. (2002) Beneficial effects of GPI 6150, an inhibitor of poly(ADP-ribose) polymerase in a rat model of splanchnic artery occlusion and reperfusion. *Shock*. **17**:222-7.

Mc Donald MC, Mota-Filipe H, Wright JA, Abdelrahman M, Threadgill MD, Thompson A, and Thiemermann C (2000) Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase on the organ injury and dysfunction caused by haemorrhagic shock. *Br J Pharmacol* **130**:843-850.

Mota-Filipe H, Sepodes B and McDonald MC (2002) The novel PARP inhibitor 5-aminoisoquinolinone reduces the liver injury caused by ischemia and reperfusion in the rat. *Med Sci Monit* **8**: BR444-BR453.

Mullane KM, Kraemer R, and Smith B. (1985) Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods*; **14**:157-67.

Novogrodsky A, Vanichkin A, Patya M, Gazit A, Osherov N, and Levitzki A. (1994). Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science* **264**: 1319-1322.

Oliver FJ, Menissier-de Murcia J, Nacci C, Decker P, Andriantsitohaina R, Muller S, de la Rubia G, Stoclet JC, and de Murcia G (1999) Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. *EMBO J* **18**:4446-54.

Ortiz LA, Champion HC, Lasky JA, Gambelli F, Gozal E, Hoyle GW, Beasley MB, Hyman AL, Friedman M, and Kadowitz PJ. (2002) Enalapril protects mice from pulmonary hypertension by inhibiting TNF-mediated activation of NF-kappaB and AP-1. *Am J Physiol Lung Cell Mol Physiol.* **282**:L1209-21.

Ortiz LA, Lasky J, Hamilton RF Jr, Holian A, Hoyle GW, Banks W, Peschon JJ, Brody AR, Lungarella G, and Friedman M. (1998) Expression of TNF and the necessity of TNF receptors in bleomycin-induced lung injury in mice. *Exp Lung Res*. **24**:721-43.

Peters-Golden M, Bailie M, Marshall T, Wilke C, Phan SH, Toews GB, and Moore BB. (2002) Protection from pulmonary fibrosis in leukotriene-deficient mice. *Am J Respir Crit Care Med*.;**165**:229-35.

Raghu G, Depaso WJ, Cain K, Hammar SP, Wetzel CE, Dreis DF, Hutchinson J, Pardee NE, and Winterbauer RH. (1991) Azathioprine combined with prednisone in the treatment of idiopathic pulmonary fibrosis: a prospective double-blind, randomized, placebo-controlled clinical trial. *Am Rev Respir Dis*.**144**:291-6.

Schutte B, Nuydens R, Geerts H, and Ramaekers F. (1998) Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. *J Neurosci Methods*. **86**:63-9.

Smith RE, Strieter RM, Phan SH, and Kunkel SL. (1996) C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. *Am J Respir Cell Mol Biol.* **15**:693-702. Review.

Southan GJ, and Szabo C. (2003) Poly(ADP-ribose) polymerase inhibitors. *Curr Med Chem.* **10:**321-40.

Suto MJ, Turner WR, AND Arundel-Suto CM (1991) Dihydroisoquinolinones: The design and synthesis of a new series of potent inhibitors of poly (ADP-ribose) polymerase. *Antican Drug Des* **6:**107-117.

Szabó C, and Dawson VL (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* **19:**287-298.

Szabó G, Bährle S, and Stumpf N (2002) Poly (ADP-ribose) polymerase inhibition reduces reperfusion injury after heart transplantation. *Circ Res* **90**: 100-106.

Venkatesan N, Punithavathi V, and Chandrakasan G. (1997) Curcumin protects bleomycin-induced lung injury in rats. *Life Sci.*61:PL51-8.

Watson CY, Whish WJD, Threadgill MD. (1998). Synthesis of 3-substituted benzamides and 5-substituted isoquinolin-1(2*H*)-ones and preliminary evaluation as inhibitors of poly (ADP-ribose) polymerase (PARP). *Bioorg Med Chem* **6**: 721-734.

Wenkert E, Johnston DBR, Dave KG. (1964) Derivatives of hemimellitic acid. A synthesis of erythrocentaurin. *J Org Chem* **29**: 2534-2542.

Wooley PH, Whalen JL, Chapman DL, Berger AE, Richard KA, Aspar DG, and Staite ND. (1993). The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen–induced arthritis in mice. *Arthritis Rheum* **36**: 1305-1314.

Xie QW, Kashiwabara Y, and Nathan C. (1994). Role of transcription factor NF-kB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**: 4705-4708.

Zhang J, Gao JX, Salojin K, Shao Q, Grattan M, Meagher C, Laird DW, and Delovitch TL. (2000) Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH_2-terminal kinase. *J Exp Med* **191**: 1017-1030,

Zhao HW, Hu SY, Barger MW, Ma JK, Castranova V, and Ma JY. (2004) Time-dependent apoptosis of alveolar macrophages from rats exposed to bleomycin: involvement of tnf receptor 2. *J Toxicol Environ Health A.* **67**:1391-406.

Zingarelli B, Cuzzocrea S, Zsengeller Z, Salzman AL, and Szabo C. (1997) Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase. *Cardiovasc Res.* **36**:205-15.

Zingarelli B, O'Connor M, and Hake PW (2003) Inhibitors of poly (ADP-ribose) polymerase modulate signal transduction pathways in colitis. *Eur J Pharmacol* **469**:183-94.

LEGENDS FOR FIGURES

Figure 1. Effect of 3-AB and 5-AIQ on lung injury and histological score. Lung injury was produced after bleomycin administration characterized by inflammatory cells infiltration, fibrosis as well as an extensive areas of collagen (**A**). The treatment with 3-AB (**B**) or with 5-AIQ (**C**) significantly reduced the extent and severity of the histological signs of colon injury. The histological score (**D**) were made by two independent observers. Figure is representative of at least 3 experiments performed on different experimental days. Data are means ± SEM of 30 mice for each group. *$p<0.01$ versus sham. °$p<0.01$ versus bleomycin.

Figure 2. The injection of bleomycin elicited an inflammatory response characterized by the accumulation of water in lung as an indicator of edema. The treatment with the two PARP inhibitors 3-AB or 5-AIQ significantly reduced the edema formation. Data are means ±s.e. means from 10 mice for each group. *$p<0.01$ versus sham. °$p<0.01$ versus bleomycin.

Figure 3. Effect of 3-AB and 5-AIQ on myeloperoxidase activity in the lung. Myeloperoxidase (MPO) actvity in the lungs of bleomycin-treated mice were significantly increased in comparison to sham-operated mice. 3-AB and 5-AIQ treatment reduced the bleomycin-induced increase in MPO activity. Data are means ±s.e. means from 10 mice for each group. *$p<0.01$ versus sham. °$p<0.01$ versus bleomycin.

Figure 4. Effect of 3-AB and 5-AIQ treatment on body weight. Body weight was recorded immediately before bleomycin administration and daily for all the experimental period. 3-AB and 5-AIQ treatment significantly prevent the loss of body weight. Data are means ±s.e. means from 30 mice for each group. *$p<0.01$ versus bleomycin.

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Figure 5. Effect of 3-AB and 5-AIQ treatment on bleomycin-induced mortality. Survival is significantly improved in 3-AB and 5-AIQ -treated mice in comparison to the high mortality rate of the bleomycin-treated mice. Data are means ±s.e. means from 30 mice for each group. *p<0.01 versus bleomycin.

Figure 6. Lung TNFα (A) and IL1β (B) production at 15 days after bleomycin administration. A significant production of cytokines was observed in the lung tissues at 15 days after bleomycin administration. Cytokine levels were significantly reduced in 3-AB and 5-AIQ -treated mice. Data are means ± SEM of 10 mice for each group. *p<0.01 versus sham. °p<0.01 versus bleomycin.

Figure 7. Immunohistochemical localization of nitrotyrosine in the lung. After bleomycin injection, positive staining for nitrotyrosine (**A**) was localized mainly in nuclei of inflammatory cells. There was a marked reduction in the immunostaining in the lungs of bleomycin-treated mice treated with 3-AB (**B**) and 5-AIQ (**C**). This figure is representative of at least 3 experiments performed on different experimental days.

Figure 8. Immunohistochemical localization of PAR in the lung. After bleomycin injection, positive staining for PAR (**A**) was localized mainly in plasma cell and lymphocytes. There was a marked reduction in the immunostaining in the lungs of bleomycin-treated mice treated with 3-AB (**B**) and 5-AIQ (**C**). This figure is representative of at least 3 experiments performed on different experimental days.

Figure 9. Typical Densitometry evaluation. Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for nitrotyrosine and PAR from lung tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. ND: not detectable. *$p<0.01$ versus sham. °$p<0.01$ versus bleomycin.

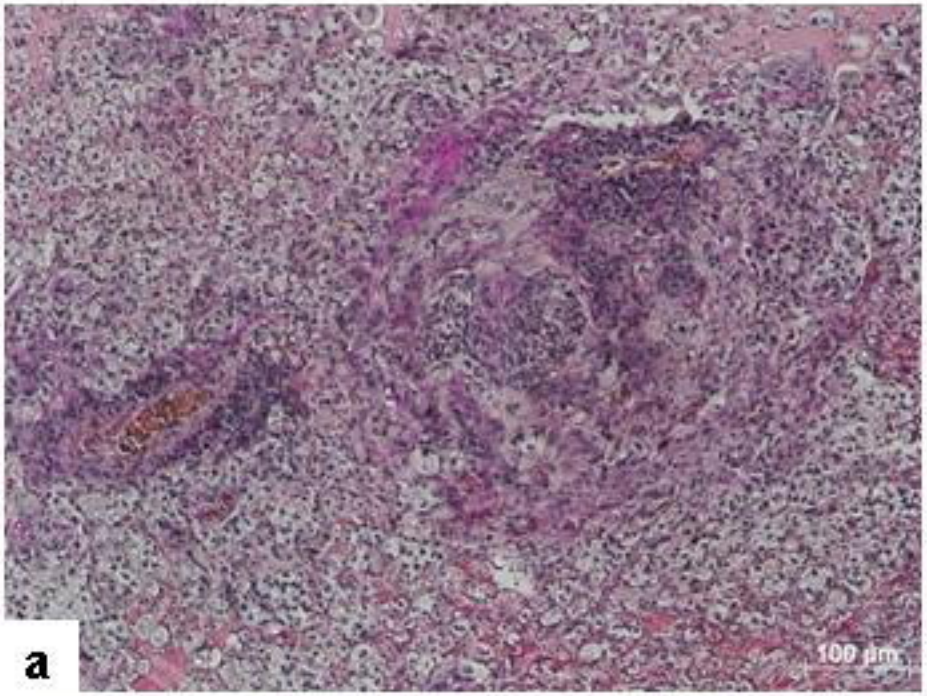
Figure 10. Effect of PARP inhibitors on apoptosis. At 15 days after bleomycin administration, lung tissues obtained from vehicle-treated mice demonstrated a marked presence of cells in the later stages of apoptosis (**A**, positive to propidium iodide) and some positive staining for Annexin V FITC (**B** index of cells that are induced to undergo apoptosis). On the contrary, lung tissues section from bleomycin-treated mice, which had been treated with 3-AB (**D**) or with 5-AIQ (**G**) demonstrate significantly less presence of cells in the later stages of apoptosis (positive to propidium iodide) and almost no positive staining necrotic for Annexin V FITC (**E**,**H** respectively). Figs **C, F** and **I** represent the staining combination of panel A-B, D-E and G-H respectively. Figure is representative of at least 3 experiments performed on different experimental days.

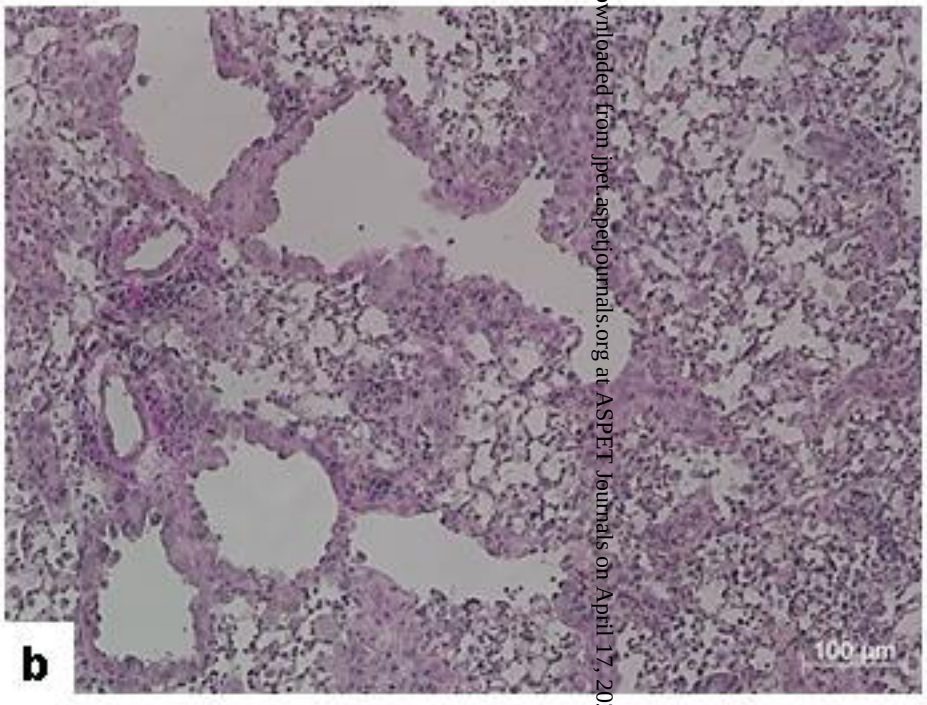
Figure 11. Representative Western blots of IκB-α degradation (**a**), as well as the densitometric analysis (**b**). Western blot analysis shows the effect of 3-AB or 5-AIQ on degradation of IκB-α in lung tissue collected at 15 days after bleomycin administration. **Sham:** basal level of IκB-α band was present in the tissue from sham-treated mice. **BLEO**: IκB-α band has disappeared in the lung tissue from bleomycin-treated mice. **BLEO + 3-AB**: IκB-α band remained unchanged in the lung tissue from bleomycin-treated mice which

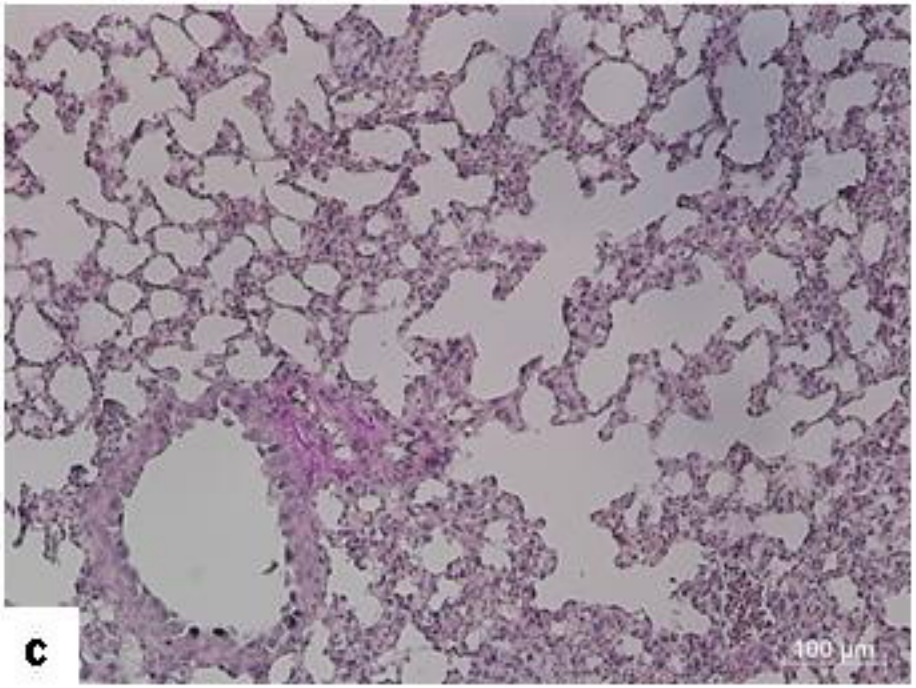
received 3-AB. **BLEO + 5-AIQ**: IκB-α band remained unchanged in the lung tissue from bleomycin-treated mice which received 5-AIQ. Immunoblotting in panel A is representative of one lung tissue out of 10 analyzed. The results in panel B are expressed as mean ± s.e.mean from 10 blots **P*<0.01 *versus* SHAM, °*P*<0.01 *versus* SCI. *p<0.01 versus sham. °p<0.01 versus bleomycin.

Figure 12. Effect of PARP inhibitors on NF-κB/DNA binding activity in mice lung. Whole extracts from bleomycin-treated mice (**BLEO**) or non-inflamed (**sham**) mice lungs were prepared as described in Materials and Methods and incubated with ^{32}P-labelled NF-κB probe. Representative EMSA of NF-κB shows the effect of 3-AB (**BLEO + 3-AB**), as well as the effect of 5-AIQ (**BLEO + 5-AIQ**) on NF-κB/DNA binding activity evaluated in lung tissues 15 days after bleomycin administration. Data illustrated are from a single experiment and are representative of 3 separate experiments.

Figure 1







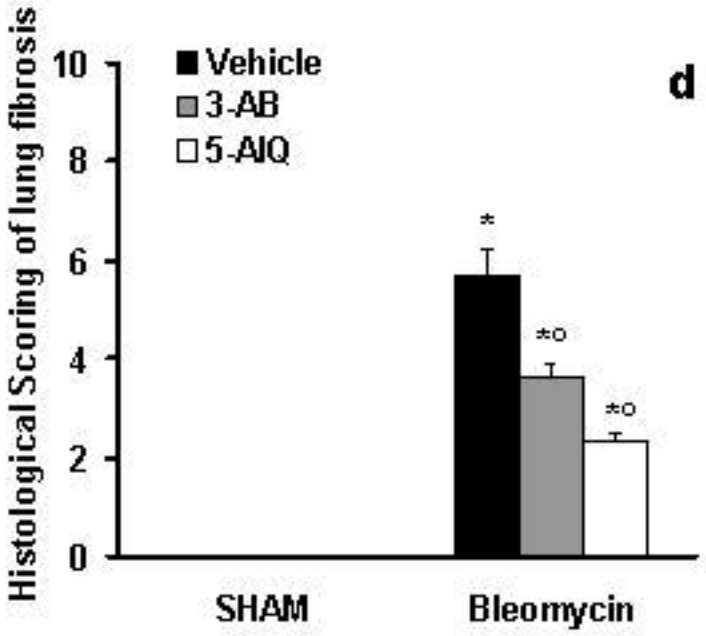


Figure 2

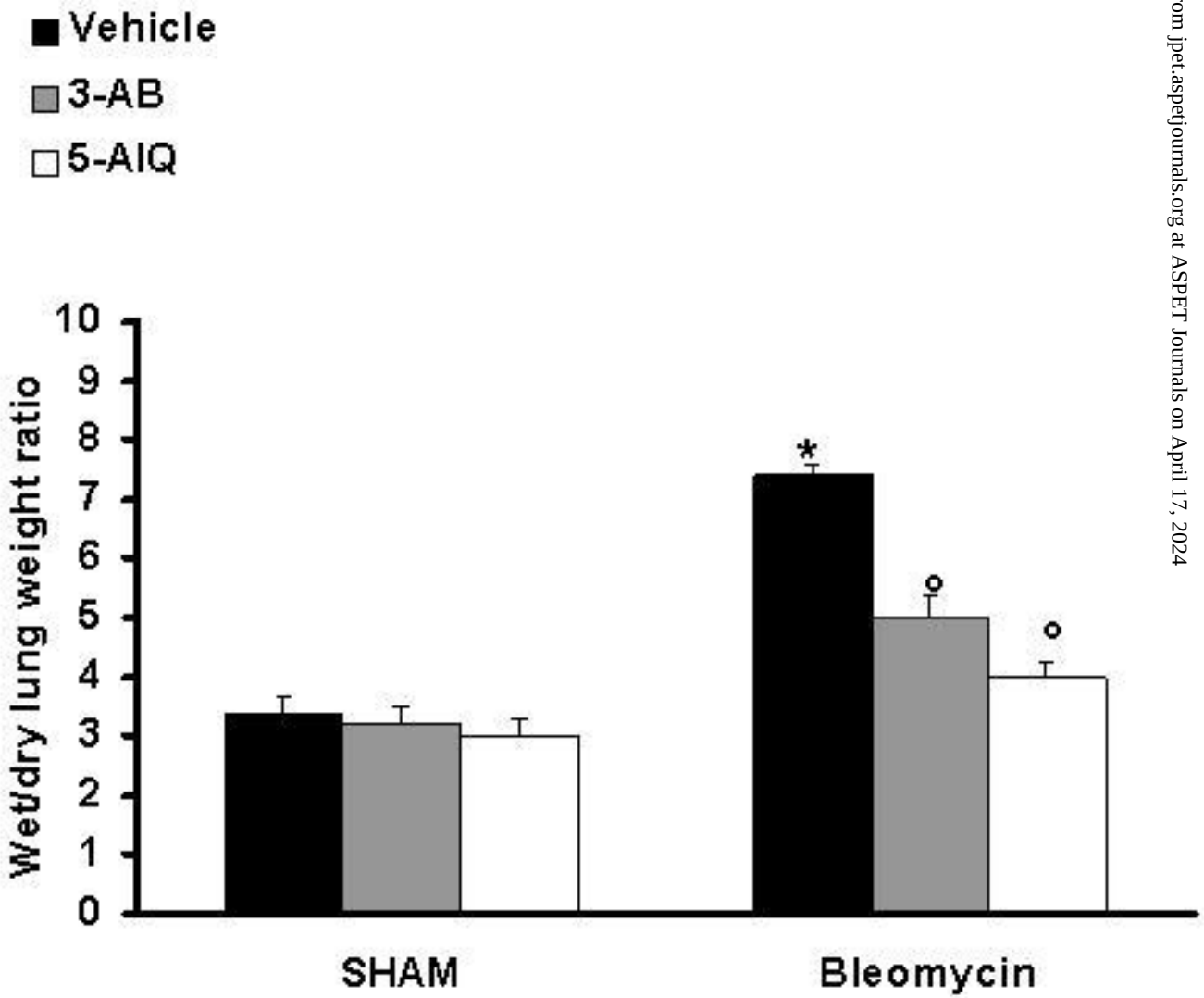


Figure 3

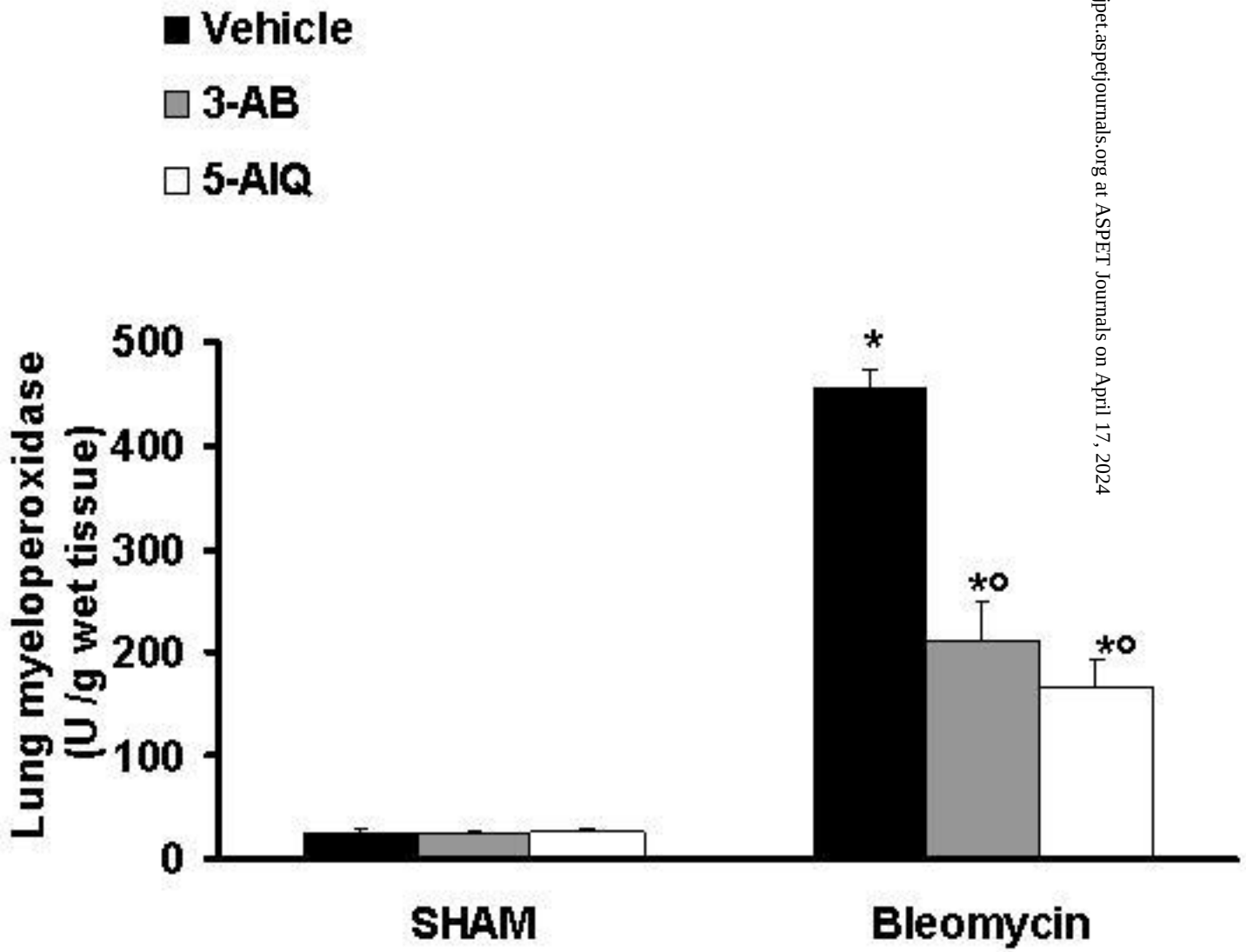


Figure 4

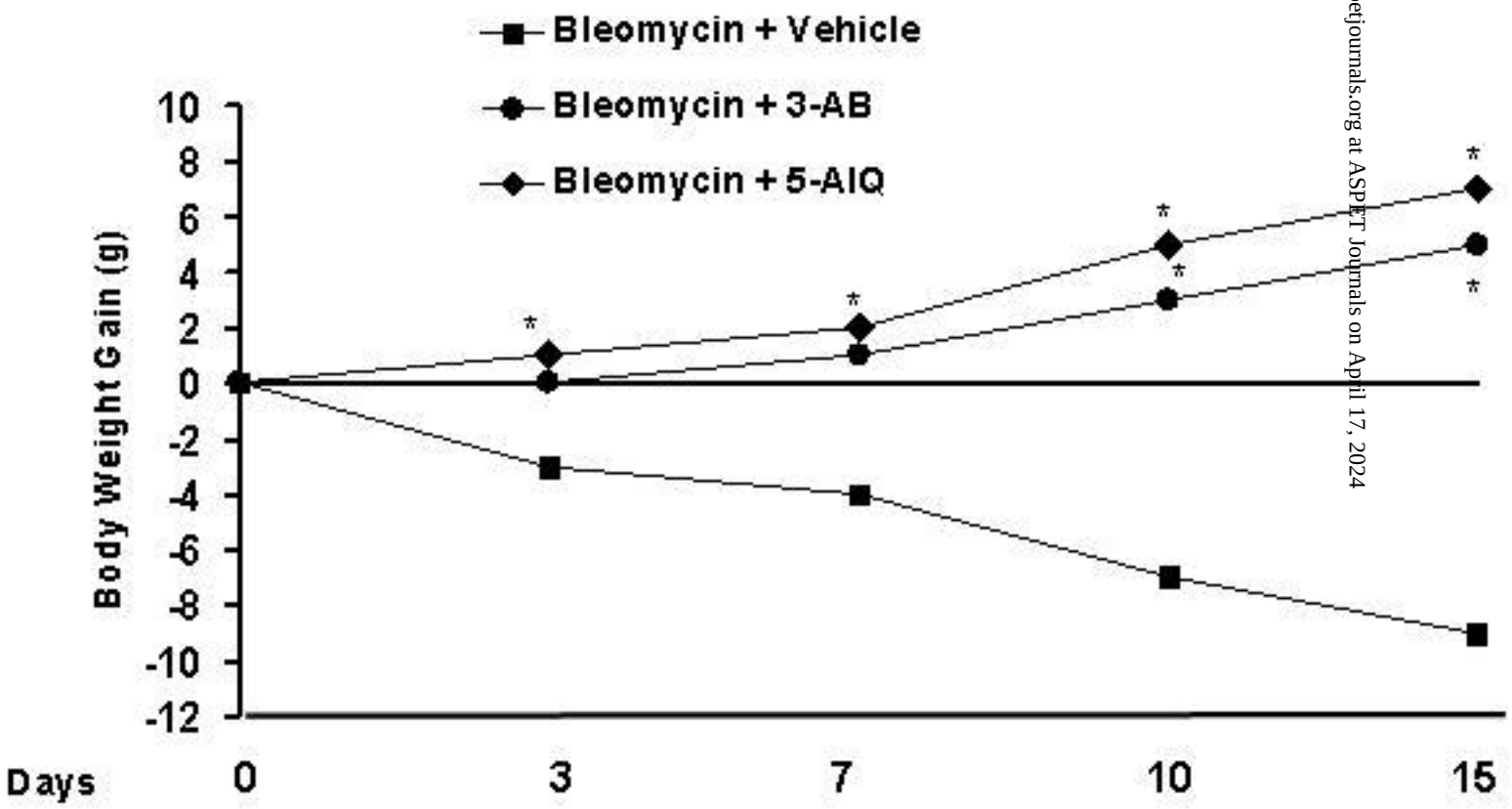


Figure 5

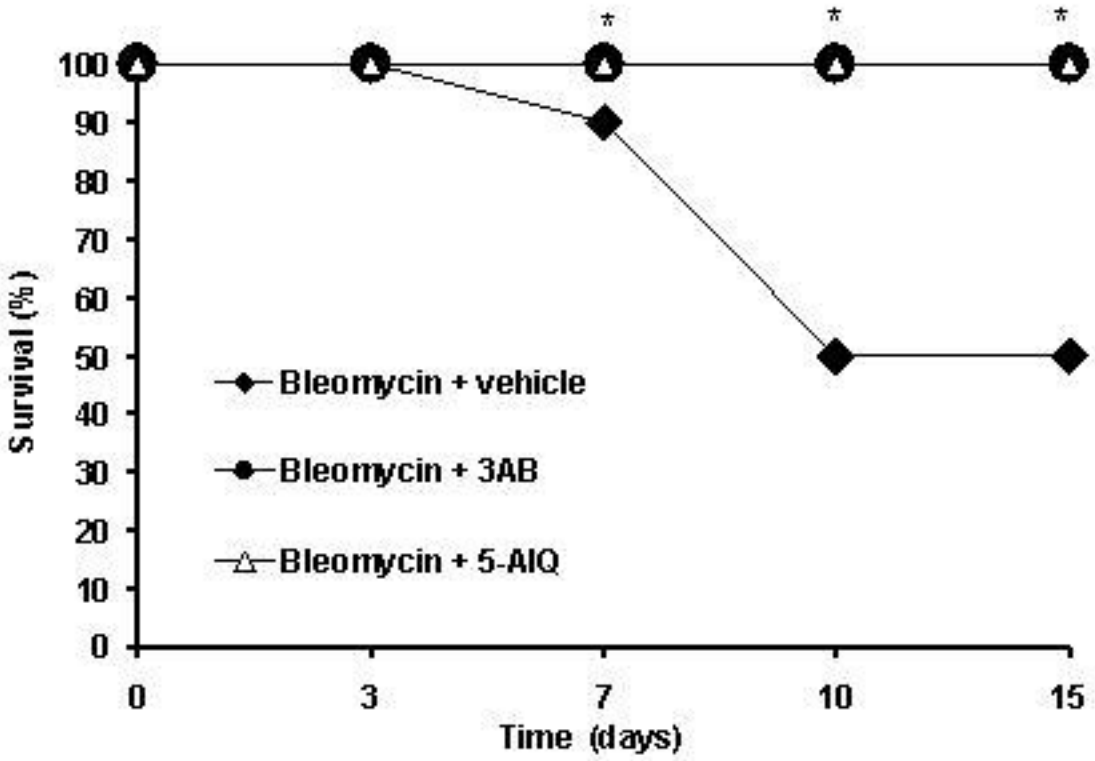
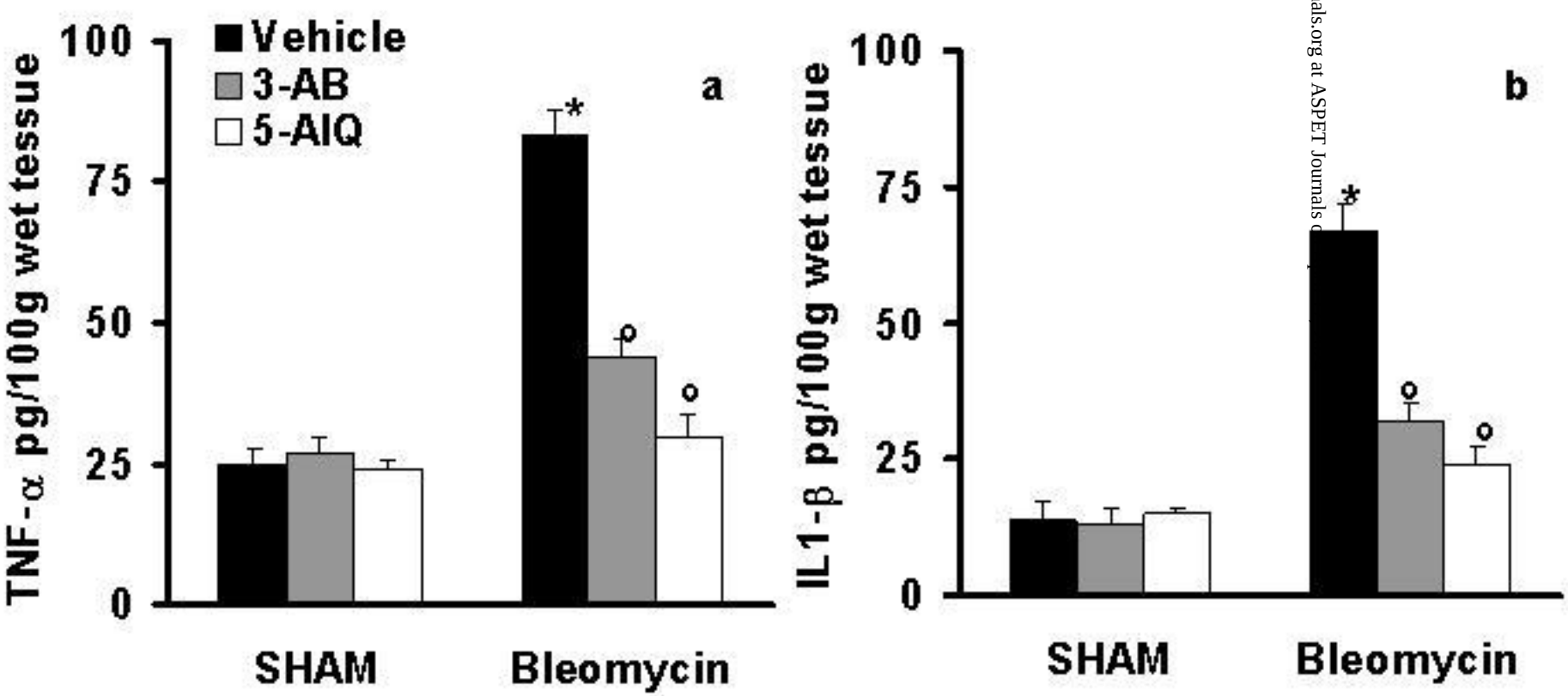
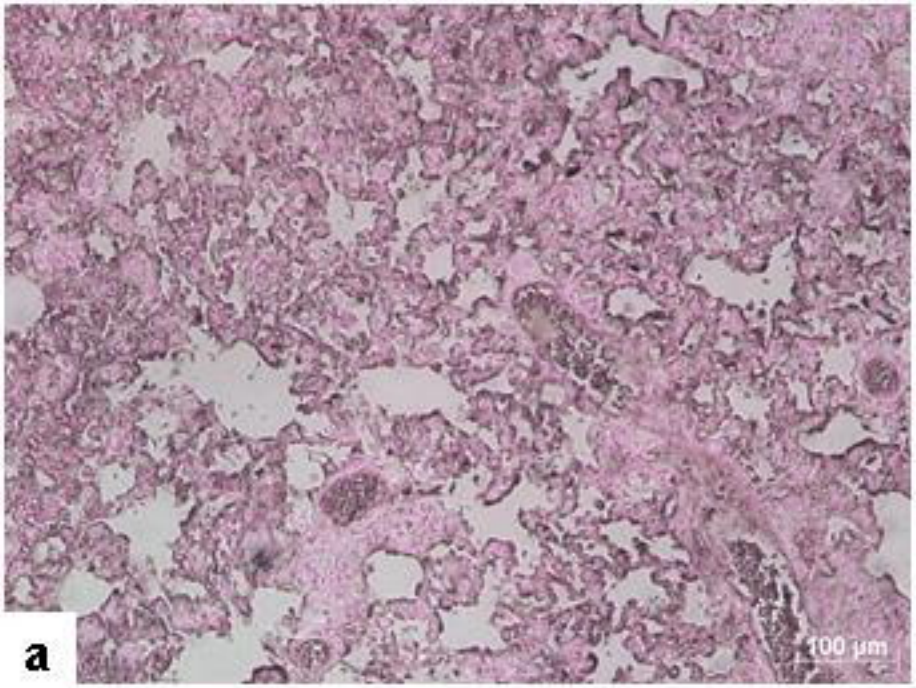
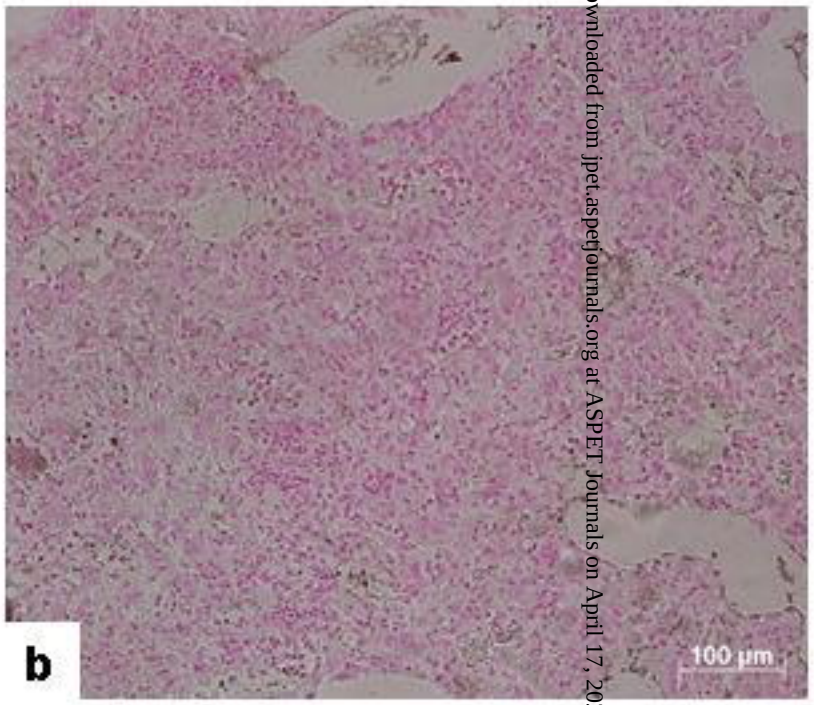


Figure 6







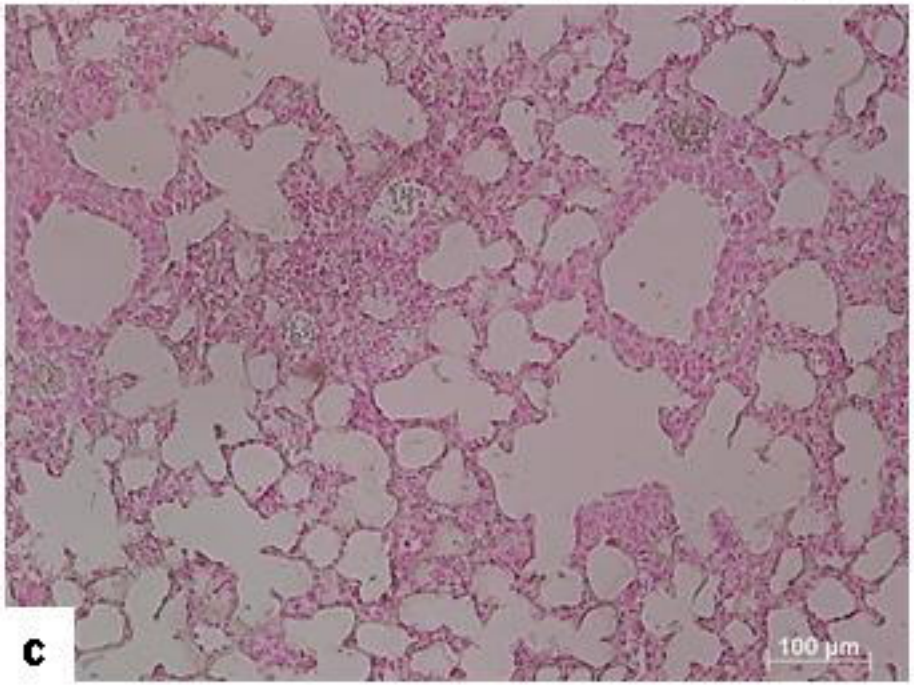
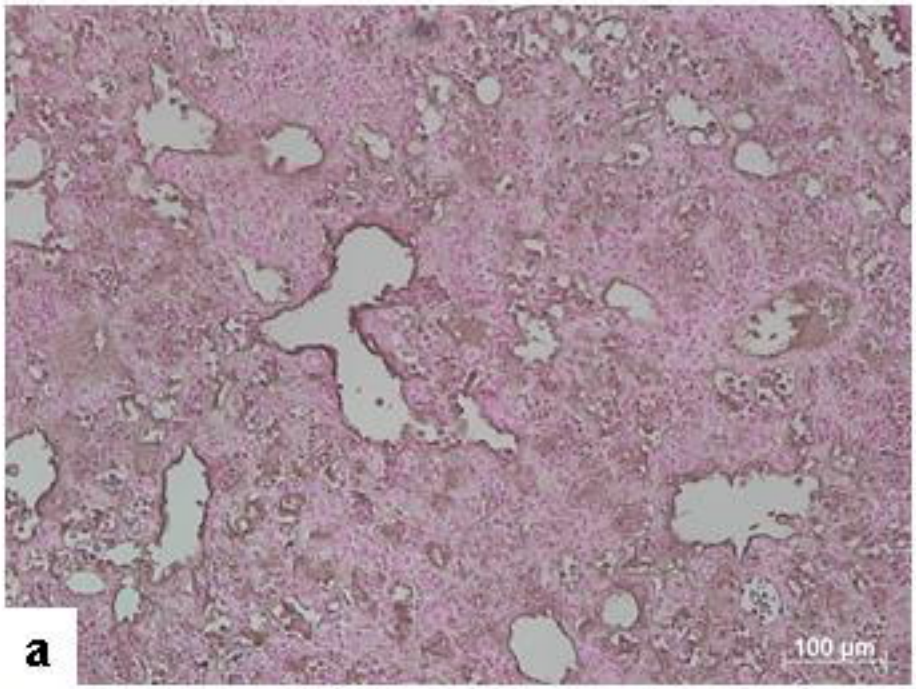
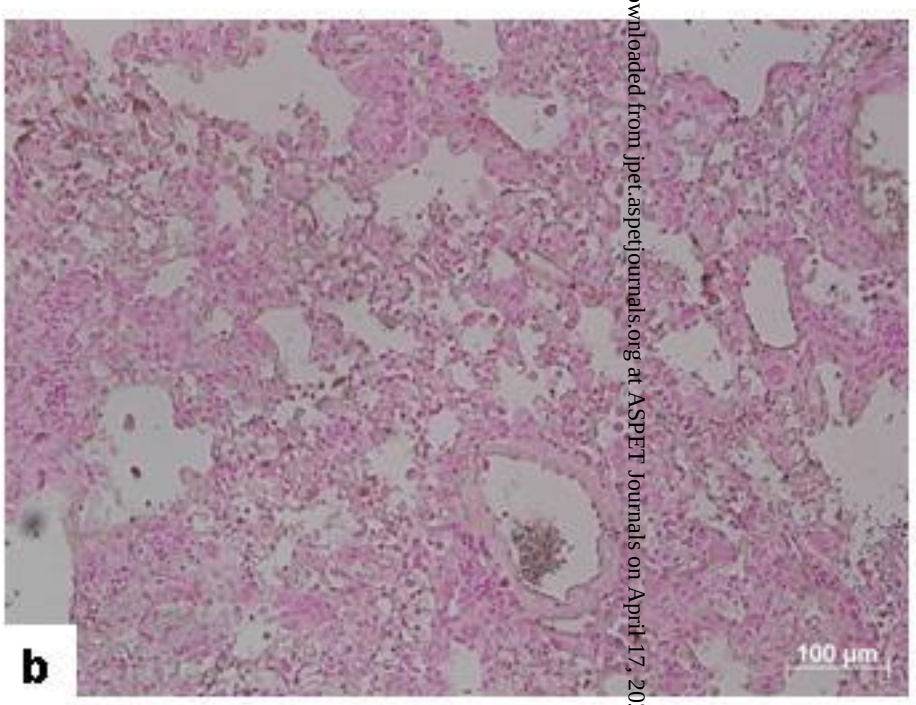


Figure 7





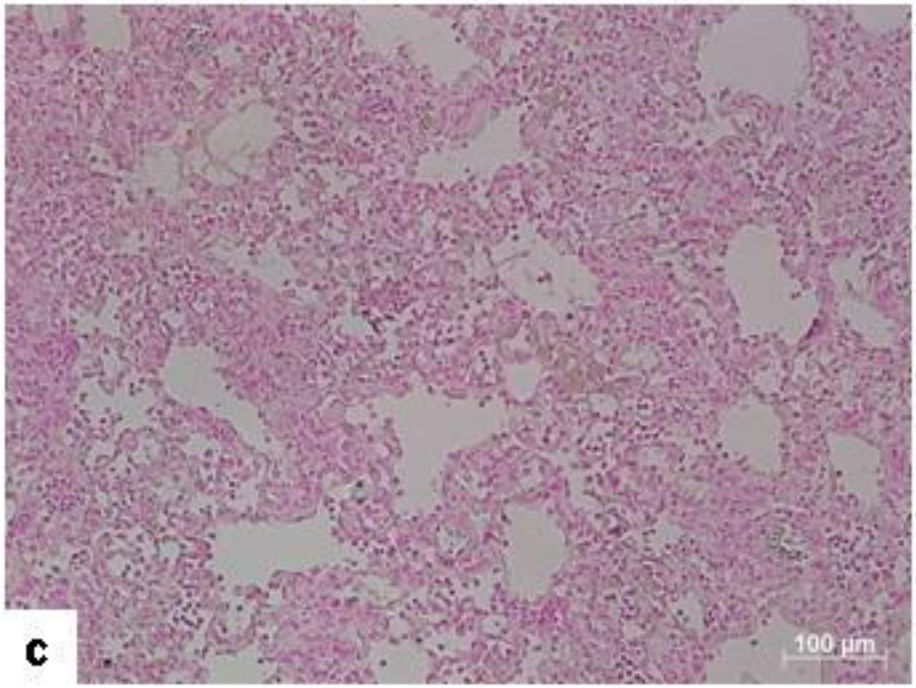


Figure 8

Figure 9

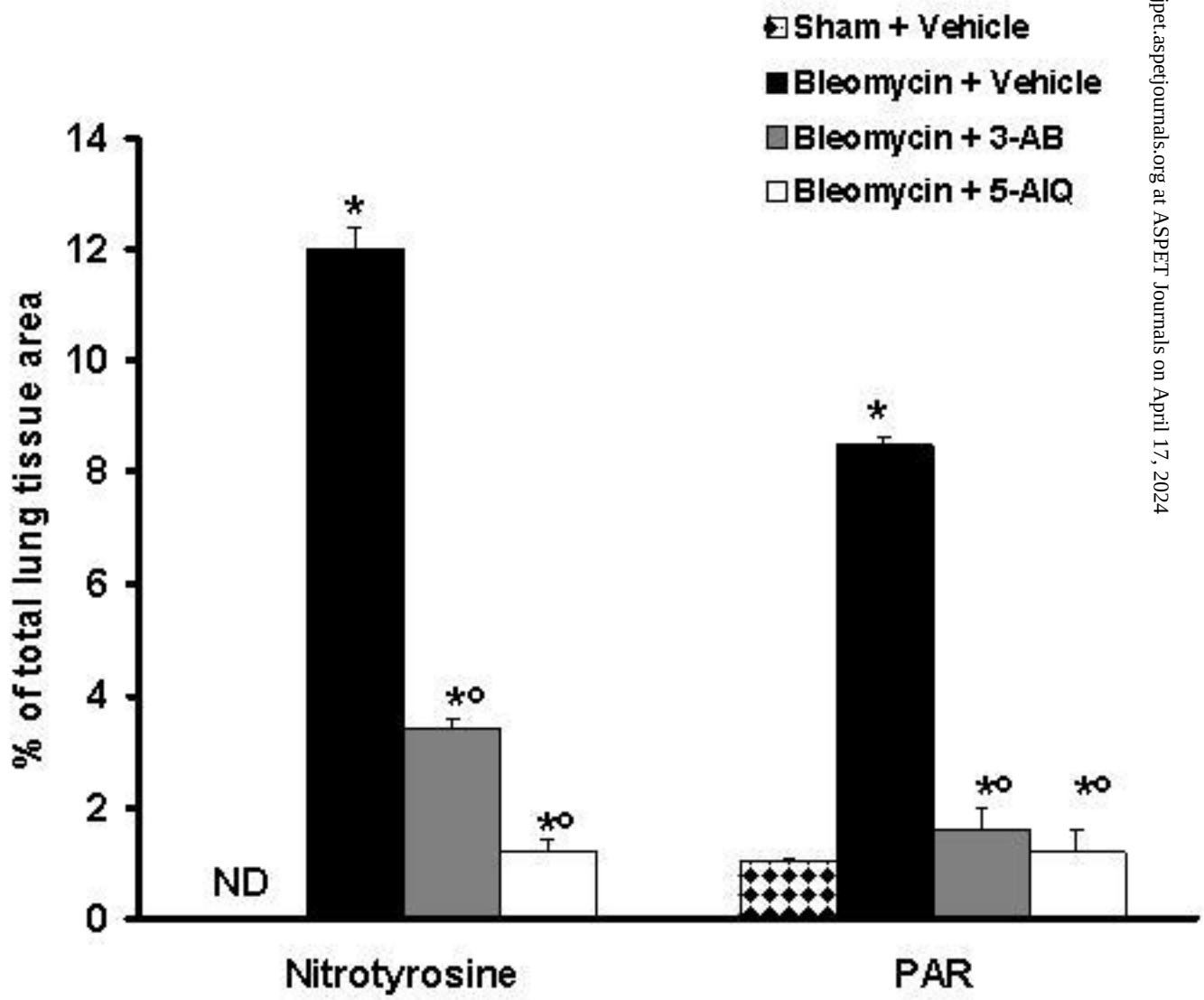


Figure 10

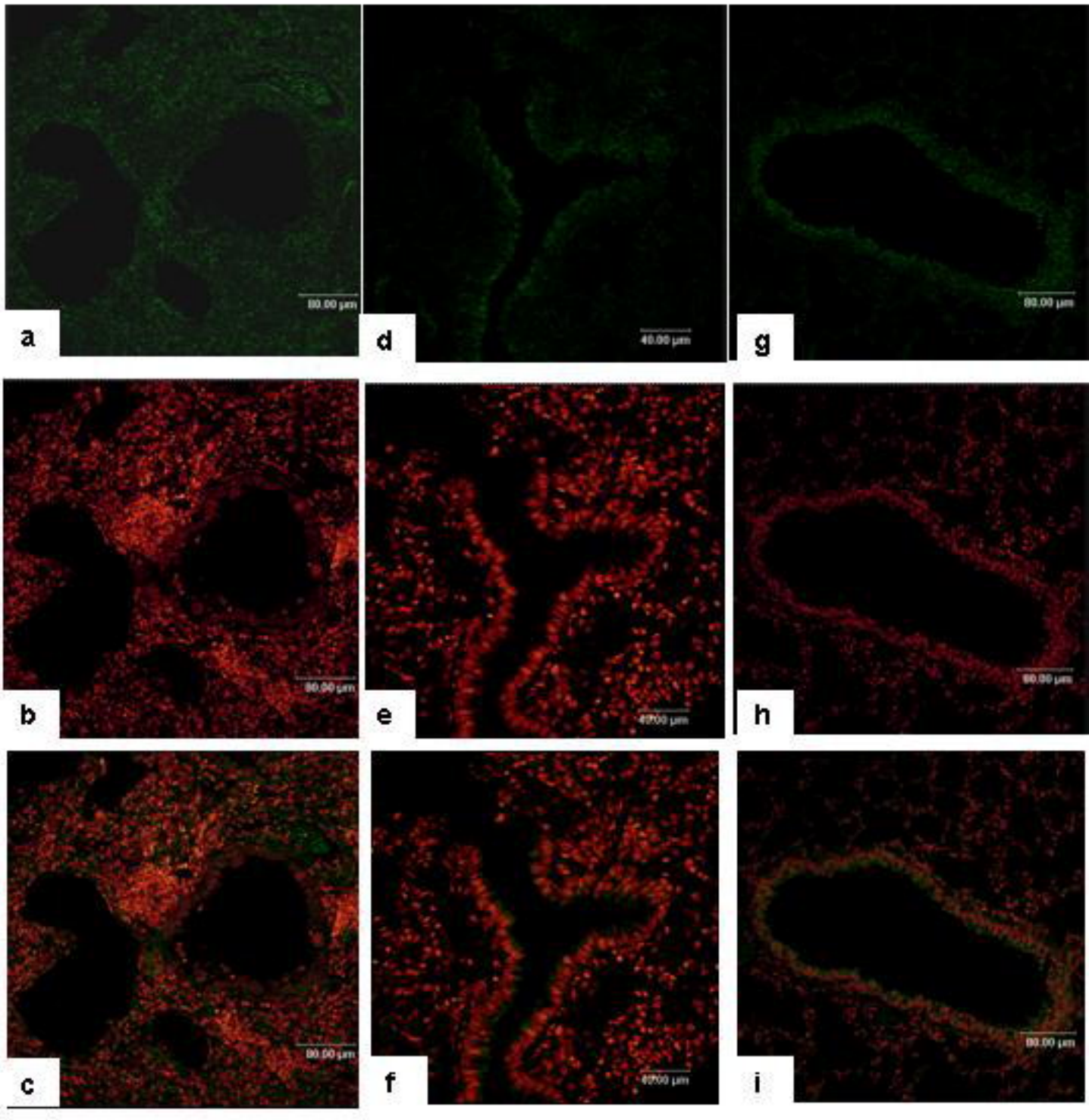


Figure 11

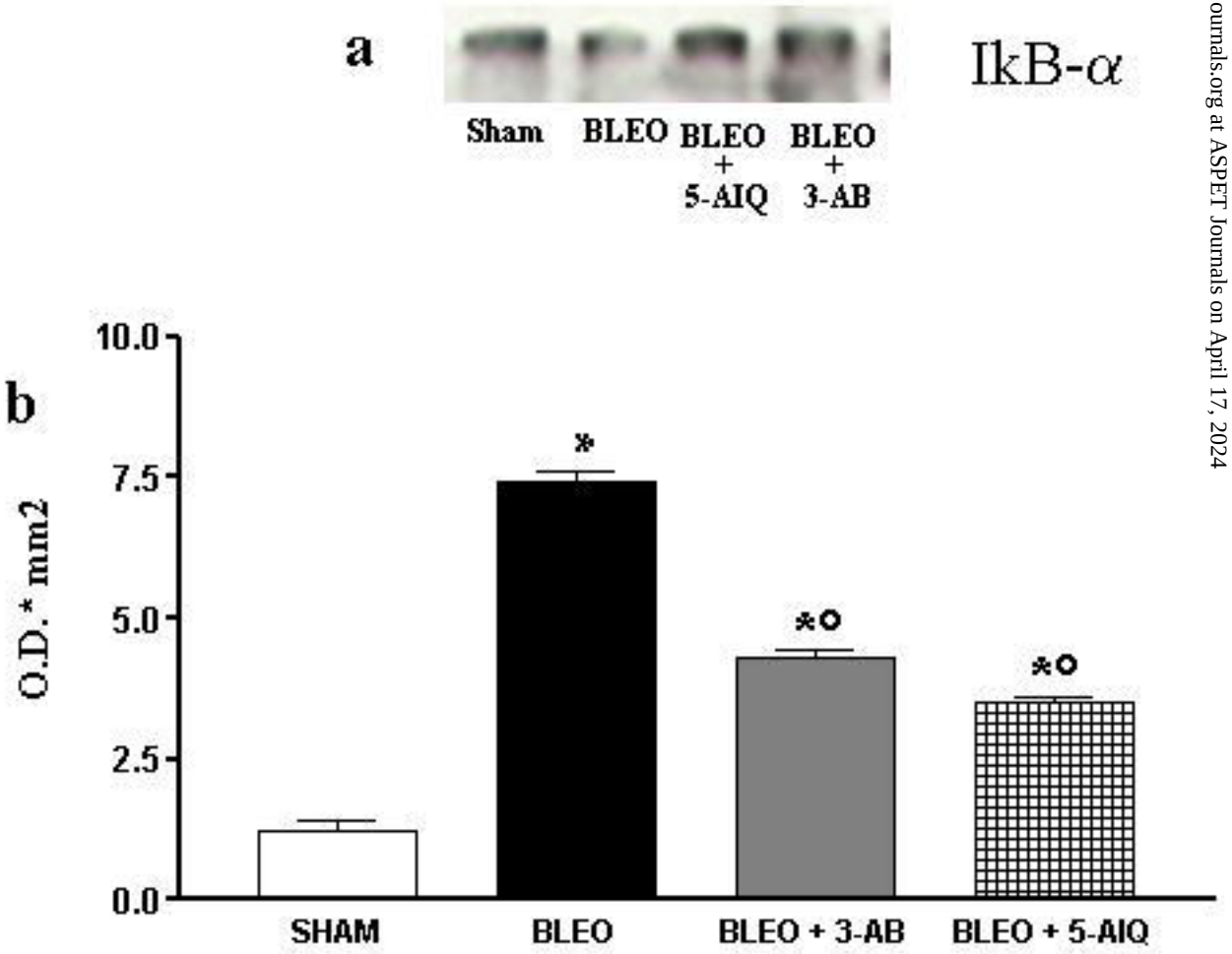


Figure 12