Proteasome Inhibition Reduces Proliferation, Collagen Expression, and Inflammatory Cytokine Production in Nasal Mucosa and Polyp Fibroblasts

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ABSTRACT

Proteasome inhibitors, used in cancer treatment for their pro-apoptotic effects, have anti-inflammatory and antifibrotic effects on animal models of various inflammatory and fibrotic diseases. Their effects in cells from patients affected by either inflammatory or fibrotic diseases have been poorly investigated. Nasal polyposis is a chronic inflammatory disease of the sinus mucosa characterized by tissue inflammation and remodeling. We tested the hypothesis that proteasome inhibition of nasal polyp fibroblasts might reduce their proliferation and inflammatory and fibrotic response. Accordingly, we investigated the effect of the proteasome inhibitor Z-Leu-Leu-Leu-B(OH)₂ (MG262) on cell viability and proliferation and on the production of collagen and inflammatory cytokines in nasal polyp and nasal mucosa fibroblasts obtained from surgery specimens. MG262 reduced the viability of nasal mucosa and polyp fibroblasts concentration- and time-dependently, with marked effects after 48 h of treatment. The proteasome inhibitor bortezomib provoked a similar effect. MG262-induced cell death involved loss of mitochondrial membrane potential, caspase-3 and poly(ADP-ribose) polymerase activation, induction of c-Jun phosphorylation, and mitogen-activated protein kinase phosphatase-1 expression. Low concentrations of MG262 provoked growth arrest, inhibited DNA replication and retinoblastoma phosphorylation, and increased expression of the cell cycle inhibitors p21 and p27. MG262 concentration-dependently inhibited basal and transforming growth factor-β-induced collagen mRNA expression and interleukin (IL)-1β-induced production of IL-6, IL-8, monocyte chemotactic protein-1, regulated on activation normal T cell expressed and secreted, and granulocyte/macrophage colony-stimulating factor in both fibroblast types. MG262 inhibited IL-1β/tumor necrosis factor-α-induced activation of nuclear factor-κB. We conclude that noncytotoxic treatment with MG262 reduces the proliferative, fibrotic, and inflammatory response of nasal fibroblasts, whereas high MG262 concentrations induce apoptosis.
shown to reduce the inflammatory response in experimental animal models of hypertension (Ludwig et al., 2009), arthritis (Palombella et al., 1998; Yannaki et al., 2010), colitis (Schmidt et al., 2010), and asthma (Althoff et al., 1999). Proteasome inhibitors have also demonstrated antifibrotic effects on animal models of cardiac fibrosis (Meiners et al., 2004), muscle atrophy (Carmignac et al., 2011), and lung and skin fibrosis (Mutlu et al., 2012).

However, none of the numerous studies undertaken, both in vitro and in vivo, have clearly established the concentrations of the proteasome inhibitor that affect cell/animal viability and modulate cell function. In fact, the effect of proteasome inhibition on cell viability in noncancerous cells has scarcely been investigated and has often been overlooked in studies reporting anti-inflammatory or antifibrotic effects of proteasome inhibitors. Furthermore, the antiproliferative, anti-inflammatory, and antifibrotic potential of proteasome inhibitors and their underlying mechanisms in the cells of patients affected by either inflammatory or fibrotic diseases have been poorly investigated. These cells may significantly differ from cancerous cells in this respect.

Chronic rhinosinusitis with nasal polyps is a chronic inflammatory disease of the sinus mucosa characterized by both tissue inflammation and remodeling (Bachert et al., 2009). Nasal polyps reveal frequent epithelial damage, a thickened basement membrane, and an edematous stroma with abundant infiltration of inflammatory cells, predominantly activated eosinophils, and fibrotic foci. Nasal polyp fibroblasts are key producers of extracellular matrix proteins, such as collagens, as well as proinflammatory cytokines and chemokines.

In addition to maintaining the tissue structure, collagens are involved in cell adhesion, chemotaxis, and migration in processes of growth, differentiation, and wound healing and also in many pathological states. All collagens have three polypeptide chains, known as α-chains. There are at least 29 human collagen types, numbered from I to XXX, which can be divided into several subfamilies on the basis of their supramolecular assemblies. Collagen types I, II, and III, among others, are fibril-forming collagens (Myllyharju and Kivirikko, 2004). Collagen I, synthesized as two procollagen chains α1 and α2, each encoded by separate genes, is a major component of the extracellular matrix. An altered profile of collagens has been reported in the airways of asthmatics (Burgess, 2009). Patients with nasal polyps and associated asthma have increased fibroblast proliferation and extracellular matrix deposition and increased fibroblast differentiation into myofibroblasts (Pawliczak et al., 2005). Collagens I and III are expressed by lung (Goulet et al., 2007) and nasal fibroblasts (Pujols et al., 2011), and their expression is increased by IL-6, IL-8, granulocyte/macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), and regulated on activation normal T cell expressed and secreted (RANTES), that recruit and activate inflammatory cells into the nasal polyp site, thus contributing to the perpetuation of inflammatory and fibrotic processes in the tissue (Xing et al., 1993; Pujols et al., 2011).

Corticosteroids, the gold standard of nasal polyp treatment, are ineffective in some patients, who will require one or even several surgical operations to remove nasal polyps (Fokkens et al., 2007). Nasal polyps are, therefore, an optimal model for researching the etiology of chronic inflammatory airway diseases, as well as potential therapeutic treatments.

In the current work, we tested the hypothesis that proteasome inhibition of nasal polyp fibroblasts might reduce their proliferation and also modulate their inflammatory and fibrotic response. To do this, we used nasal fibroblasts obtained from patients with nasal polyps and asthma and control subjects to characterize the effects of proteasome inhibition on the inflammatory and fibrotic cellular response. More specifically, we aimed to examine the effect of the proteasome inhibitor Z-Leu-Leu-Leu-βOH (MG262), which, like bortezomib, is a boronic acid peptide that selectively and reversibly inhibits the chymotryptic activity of the proteasome, on cell viability and proliferation and the production of collagen and inflammatory cytokines in nasal polyp and nasal mucosa fibroblasts.

### Materials and Methods

**Reagents.** Duleboce’s modified Eagle’s medium (DMEM) was obtained from Lonza (Verviers, Belgium), and fetal bovine serum (FBS) was from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Trypsin-EDTA, penicillin, streptomycin, HEPES, small interfering RNA (siRNA), and all other transfection reagents were purchased from Life Technologies (Paisley, UK), and amphotericin B was from Bristol-Myers Squibb (Middlesex, UK). MG262, IκB kinase (IKK) inhibitor III (4′2′-aminophenylamino-1′,8-dimethylimidazo[1,2-a]quinoxaline (BMS-345541), anthem[a,9-c-pyrazole-6(2H)-one (S600125), 4′(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminothenyl]butadiene (U0126), wortmannin, and caspase inhibitor I [Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-FMK)] were obtained from Selleckchem (Houston, TX). All drugs were dissolved in dimethyl sulfoxide at least at 500 μM and further diluted in cell culture medium. TGF-β, IL-1β, and tumor necrosis factor-α (TNF-α) were used.
purchased from R&D Systems Europe Ltd (Abingdon, Oxfordshire, UK). Pifithrin-α (2-[2-(iminomethyl)-4,5,6,7-tetrahydrobenzothiazol-3-yl]-1-p-tolyethanolamine, HBr) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Subjects. Human nasal mucosa was obtained from nine subjects [44.6 (mean) ± 4.6 (S.E.M.) years; five men] without any history of nasal or sinus disease who underwent nasal corrective surgery for turbinate hypertrophy or septal dismorphism. Nasal polyps were obtained from 12 patients [47.5 (mean) ± 3.1 (S.E.M.) years; five men] with diagnosis of nasal polyposis and asthma undergoing functional endoscopic sinus surgery. None of the patients had suffered from upper respiratory infection for at least 2 weeks before surgery. The diagnosis of nasal polyposis and asthma was based on established criteria as reported previously (Pujols et al., 2011). All subjects agreed to participate in the study, which was approved by the Ethics Committee of Hospital Clinic, Barcelona, Spain.

Fibroblast Culture. Fragments (approximately 3×3 mm) of the nasal tissues were placed in six-well culture dishes in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B, in a humidified atmosphere at 37°C and 5% CO₂. Fibroblasts were isolated from the tissue fragments through adhesion and migration on the plastic surface and further characterized by immunostaining of cytokeratin and vimentin as reported previously (Pujols et al., 2011). In all experiments, cells were growth-arrested by incubation with serum-free medium for 18 to 24 h before drug incubation. The experiments were carried out between passages 4 and 8.

Small Interfering RNA Silencing. Thirty to 50% confluent nasal fibroblasts were transfected with 20 nM of either c-Jun Silencer Select predesigned siRNA (Life Technologies) or Silencer Select negative control siRNA (Life Technologies) duplexes in complex with Lipofectamine RNAiMAX (Life Technologies) in OptiMem I (Life Technologies) (1.5 μl/well for 24-well plates; 7.5 μl/well for six-well plates; 15 μl/well for 6-cm dishes). Medium was changed 18 h after transfection. Forty-eight hours after transfection, cells were incubated with MG262 (10 nM) for an additional 48 h.

Trypan Blue Exclusion Assay. Fibroblasts were transfected and treated with MG262 as indicated above. The cell culture supernatants were collected, and the trypan blue dye solution (0.4%) was added to the cell suspensions. The total number of dead cells was determined by using a standard hemocytometer procedure. Dead cells were seen as blue (permeable to dye because of disruption of the cell membrane).

Cell Viability Assays. Cells were plated in 96-well culture plates (1–2×10⁴ cells/well). The next day, cells were growth-arrested as indicated above and incubated with cell medium supplemented with 0.5% FBS with or without the simultaneous addition of the proteasome inhibitor MG262 (0.1–1000 nM) for 24, 48, and 72 h or with the proteasome inhibitor bortezomib (0.1–1000 nM) for 48 h. In some experiments, the caspase inhibitor z-VAD-FMK was added (50 μM) for 1 h before incubation with MG262. After treatment, cell viability was determined by using the colorimetric Cell Proliferation Kit II (XTT; Roche Diagnostics, Mannheim, Germany). This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active (viable) cells. Absorbance was measured on a microplate spectrophotometer at 490 nm.

Analysis of Mitochondrial Membrane Potential. Fibroblasts were incubated with cell medium supplemented with 0.5% FBS with or without the simultaneous addition of MG262 (50, 500, and 1000 nM) for 24 h. After treatment, the cells were incubated with the fluorescent mitochondrial dye 3,3′-dihexyloxacarbocyanine iodide (DiOC₆(3) 40 nM) for 30 min at 37°C. The cell culture supernatant was then collected and combined with the trypanized (0.05% trypsin-0.02% EDTA) adherent cells. The cells were then centrifuged, resuspended in phosphate-buffered saline (PBS), and incubated for 15 min with a violet-fluorescent reactive dye (Live/Dead fixable dead cell stain kit; Life Technologies). The percentages of viable cells and those exhibiting loss of DiOC₆ uptake were determined by fluoros-
Likewise, comparisons between two groups were made with the Kruskal-Wallis test. If data were normally distributed and had similar variances or the Mann-Whitney U test if the variable distributions were not normal and/or had different variances. The concentration (nM) of drug that resulted in 50% inhibition (IC$_{50}$) of the effect provoked by the stimulus was calculated with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL) and GraphPad Prism software. Statistical significance was established as $p < 0.05$.

**Results**

**Effect of MG262 on Fibroblast Viability.** It is well known that the efficacy of proteasome inhibitors, in particular bortezomib, in cancer relies on their capacity to induce cell death (Meiners et al., 2008). However, the effect of proteasome inhibitors on cell viability in noncancerous cells has been less widely investigated and remains controversial (Fin-eschi et al., 2006; You and Park, 2011). In an attempt to discern the concentrations and incubation times with the proteasome inhibitor MG262 that affect the viability of nasal fibroblasts, we carried out concentration-response and time-course experiments with the proteasome inhibitor MG262 and analyzed the viability of nasal fibroblasts thereafter by using the XTT metabolic assay. As shown in Fig. 1A, the treatment of nasal fibroblasts with MG262 for 24 h provoked a significant concentration-dependent reduction in cell viability in both nasal mucosa and nasal polyp fibroblasts. The maximal suppression reached approximately 40% in both cases. A marked concentration-dependent decrease in cell viability was found after 48 h (Fig. 1B) and 72 h (Fig. 1C) of MG262 treatment. No differences in IC$_{50}$ values (median, 25–75th percentiles, in nM) were found between nasal mucosa (48 h, median 11, 10–23 nM; 72 h, median 4, 3–6 nM) and nasal polyp fibroblasts (48 h, median 7, 5–35 nM; 72 h, median 4, 3–8 nM; not significant). A significant decrease in cell viability was also found after 48 h of fibroblast treatment with the proteasome inhibitor bortezomib (Fig. 1D). IC$_{50}$

![Fig. 1. Effect of MG262 on nasal fibroblast viability. Nasal mucosa (solid lines) and nasal polyp (dashed lines) fibroblasts were incubated with DMEM supplemented with 0.5% FBS with/without the simultaneous addition of MG262 for 24 h (A), 48 h (B), or 72 h (C) or with bortezomib for 48 h (D). Cell viability was then determined by measuring (490 nm) the formation of a soluble formazan salt by metabolic active (viable) cells using the XTT assay. Data represent the median and interquartile range of at least five independent experiments from different patients. $^*$, $p < 0.05$ versus untreated cells.](https://jct.aspetjournals.org/)

Cruz Biotechnology, Inc., Santa Cruz, CA), total retinoblastoma (Rb; Santa Cruz Biotechnology, Inc.), cyclin D1 (Santa Cruz Biotechnology, Inc.), p53 (Ab-5; Thermo Fisher Scientific, Waltham, MA), phosphorylated (Ser607/611) Rb (Cell Signaling Technology, Danvers, MA), phosphorylated (Thr180/Tyr182) p38 MAPK, and phosphorylated (Ser21/Ser42) p38 MAPK (Cell Signaling Technology), cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Danvers, MA), phosphorylated (Ser32) IκB-α (Calbiochem), and β-actin (Sigma-Aldrich). After washing with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed, and visualized by enhanced chemiluminescence reaction (SuperSignal West Pico; Life Technologies). Light emission was detected by a charge-coupled device camera (LAS-3000; Fujifilm, Tokyo, Japan).

**Immunocytochemistry.** Fibroblasts were treated with cell medium supplemented with 10% cFBS in the absence or presence of 500 nM MG262 for 1 h, and then stimulated with TNF-α or IL-1β (100 ng/ml each) for 30 min. The cells were then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100/1% BSA, incubated with anti-p65 antibody (Santa Cruz Biotechnology, Inc.), washed in PBS, incubated with secondary antibody conjugated to Alexa 488, mounted in Prolong Gold aqueous mounting medium (Life Technologies), and visualized by fluorescence microscopy (DMI6000B; Leica, Wetzlar, Germany). Photographs were taken with a Leica DF350 FX digital camera and processed with LAS AF software (Leica).

**Statistical Analysis.** Results are expressed as median and 25th to 75th percentiles. Multiple comparisons, such as concentration-response experiments, were analyzed by using one-way analysis of variance with Tukey/Dunnett post hoc analysis if variables were normally distributed and had similar variances, the latter calculated by using the Barlett’s test or the Levene’s test. If the variable distributions were not normal and/or had different variances, the Kruskal-Wallis with Dunn’s post hoc analysis was used instead. Likewise, comparisons between two groups were made with the $t$ test.
values for bortezomib did not either differ between nasal mucosa (median 36, 10–191 nM) and polyp fibroblasts (median 24, 17–67 nM; not significant).

**Effect of MG262 on Apoptosis.** Because we found that MG262 reduced the viability of nasal fibroblasts and proteasome inhibitors are known to provoke tumor cell death via the induction of apoptosis (Hideshima et al., 2001), we reasoned that the cell death of nasal fibroblasts provoked by MG262 would also involve apoptosis. The initial apoptotic pathways end at the point of the execution phase, considered the final pathway of apoptosis, where the activation of executioner caspsases (of which caspase-3 is the most important) provokes the cleavage of various substrates, such as PARP, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Elmore, 2007). To elucidate whether MG262 induced nasal fibroblast apoptosis, fibroblasts were either preincubated with the caspase inhibitor z-VAD-FMK (50 µM) before incubation with MG262 or were not treated, and cell viability was analyzed by the XTT assay. As shown previously, incubation with high concentrations of MG262 for 48 h significantly reduced fibroblast viability compared with nontreated cells (Fig. 2A). More importantly, z-VAD-FMK partially prevented the reduction of fibroblast viability provoked by MG262, suggesting that the decrease in cell viability provoked by long-term exposure with MG262 involves apoptosis.

The loss of mitochondrial membrane potential is one of the earliest events that initiates the classic intrinsic pathway of apoptosis (Elmore, 2007), and proteasome inhibitors are known to induce this event in cancer cells (Ling et al., 2003b; Domingo-Doménech et al., 2008). We therefore aimed to investigate whether MG262 provoked the loss of mitochondrial membrane potential in nasal fibroblasts. As shown in Fig. 2B, the incubation of fibroblasts with MG262 for 24 h caused a significant concentration-dependent loss of mitochondrial membrane potential. We then explored whether MG262 provoked the activation of caspase-3 and that of its substrate PARP in nasal fibroblasts, as occurs after proteasome inhibition of tumor cells (Hideshima et al., 2001; Ling et al., 2003a,b). Treatment with 50 nM MG262 for 24 h did not result in the expression of cleaved caspase-3, or its substrate PARP, in any fibroblast line (Fig. 2C). At 24 h, expression of cleaved caspase-3 was found only at the highest concentration of MG262 (1000 nM). Expression of cleaved caspase-3 and PARP was detected after cell incubation with 50 nM MG262 for 48 h (Fig. 2C).

Induction of the JNK MAPK stress pathway is another reported mechanism by which proteasome inhibitors provoke apoptosis of tumor cells (Hideshima et al., 2003; Yang et al., 2004; Meiners et al., 2008). The activity of JNK and other MAPKs is endogenously controlled by different MAPK phosphatases, such as MKP-1 (Liu et al., 2007). MKP-1 is degraded through the proteasome and is known to be activated by proteasome inhibitors in cancer cells (Small et al., 2004). We hypothesized that MG262 would also activate the JNK pathway and MKP-1 in nasal fibroblasts. Accordingly, treatment of nasal fibroblasts with MG262 (50 nM) markedly induced c-Jun phosphorylation 6 and 24 h after administra-

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**Fig. 2.** Effect of MG262 on nasal fibroblast apoptosis. A, fibroblasts were incubated with DMEM supplemented with 0.5% FBS with or without z-VAD-FMK (50 µM) for 1 h before MG262 exposure for 48 h. Cell viability was measured by the XTT assay as indicated in Fig. 1. *, $p < 0.01$ versus 0% FBS. †, $p < 0.01$ versus 0.5% FBS. ‡, $p < 0.01$ versus z-VAD-FMK. B, fibroblasts were incubated with 0.5% FBS-supplemented DMEM with and without the simultaneous addition of MG262 for 24 h. Cells were then incubated with the fluorescent mitochondrial dye DiOC₆ (40 nM) and stained with a violet-fluorescent reactive dye (Live/Dead fixable dead cell stain kit), and the percentages of viable cells exhibiting loss of mitochondrial membrane potential (ΔΨm) was determined by FACS. *, $p < 0.05$ compared with untreated (C) cells. A and B show the median (bars) and interquartile range (whiskers) of five independent experiments from different patients. C, fibroblasts were incubated with 0.5% FBS-supplemented DMEM with/without the simultaneous addition of MG262 for 24 and 48 h. Cellular lysates were then obtained, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, and membranes were incubated with primary antibodies against cleaved caspase-3, PARP, and β-actin. Images are representative Western blots of three independent experiments from different patients.
tion (Fig. 3, A and B, top) and also induced MKP-1 expression (Fig. 3, A and B, bottom). We then examined the involvement of c-Jun activation on the proapoptotic effect of MG262 on our cells by silencing c-Jun expression using siRNA techniques. We first demonstrated that MG262-induced c-Jun phosphorylation was abrogated in c-Jun siRNA-transfected cells (Fig. 3C). It is noteworthy that the induction of cell death provoked by the exposure of nasal fibroblasts to 10 nM MG262 for 48 h was significantly abrogated in c-Jun siRNA-transfected cells (Fig. 3, D and E), which indicates that the increase in cell death provoked by long-term exposure with MG262 involves activation of the JNK/c-Jun pathway.

**Effect of MG262 on the Cell Cycle.** Exposure of cancer cells to proteasome inhibitors causes cell-cycle arrest (Ling et al., 2003a; Codony-Servat et al., 2006; Domingo-Domènech et al., 2008). We investigated whether MG262 also caused cell-cycle arrest in nasal fibroblasts. As expected, the treatment of growth-arrested fibroblasts with a cell growth stimulus (5% FBS) markedly increased the S-phase and G2/M population and, consequently, decreased the G0/G1 population (Fig. 4A; Table 1). MG262 (10 nM), added simultaneously to the stimulus, provoked the complete arrest of cell cycle progress compared with 5% FBS-treated cells. Thus, MG262-treated cells had a percentage of cells in G0/G1, S, and G2/M similar to that of FBS-deprived cells, indicating that MG262 did not allow cells to progress into the S phase. At this concentration, MG262 did not significantly increase the sub-G0/G1 population associated with cell death, compared with both FBS-deprived and 5% FBS-treated cells (Fig. 4A). MG262 caused cell accumulation at S and G2/M in asynchronized (nongrowth-arrested) cell cultures (data not shown). Confirming the propidium iodide staining findings (Fig. 4A), MG262 (10 nM) completely abolished cell proliferation (DNA replication), the percentage of proliferating cells being similar to that of FBS-deprived cells (Fig. 4B; Table 1).

Progression through the G1 cell-cycle restriction point is controlled by the phosphorylation status of Rb. Rb phosphorylation induced by cyclin-dependent kinase (CDK) complexes (cyclin D-CDK4/6 first, followed by cyclin E-CDK2) provokes Rb inactivation, thus permitting the release of the transcription factor E2F and cell-cycle progression (Poznic, 2009). CDK activity is negatively regulated by CDK inhibitors, including p21 and p27 (Sherr and Roberts, 1999). Because we found that MG262 provoked a cell-cycle arrest of nasal fibroblasts that did not allow cells to progress into the S phase, we hypothesized that some of the cell-cycle proteins that regulate the G1-S transition might be affected by MG262. As ex-
pected, the incubation of nasal fibroblasts with 5% FBS for 6 h, and more particularly for 24 h, provoked hyperphosphorylation of Rb compared with serum-deprived cells (Fig. 5, A and B). MG262 treatment, even at the lowest concentration tested (5 nM), completely inhibited Rb phosphorylation, in keeping with the findings of the blockade of cell-cycle progression by MG262 (Fig. 4). In addition, the incubation of fibroblasts with 5% FBS for 6 h significantly increased the expression of the positive regulatory cell-cycle protein cyclin D1, and MG262 increased (without statistical significance) cyclin D1 expression, compared with FBS-treated cells (Fig. 5C). MG262 also provoked a time- and concentration-dependent increase in the expression of the cell-cycle inhibitors p21 and to a lesser extent that of p27 (Fig. 5, D and E).

**Effect of MG262 on Collagen mRNA Expression.** Once the effect of MG262 on the cell cycle and viability of nasal fibroblasts had been characterized, we aimed to determine the capacity of MG262 to modulate, and more specifically to decrease, their fibrotic and inflammatory response. One of the first studies assessing the antifibrotic potential of proteasome inhibitors reported a decrease in collagen mRNA expression after proteasome inhibition of human dermal fibroblasts (Fineschi et al., 2006). We therefore investigated whether MG262 also reduced collagen expression in nasal fibroblasts. In agreement with our previous studies (Pujols et al., 2011), the profibrogenic stimulus TGF-β/H9252 (5 ng/ml) induced the mRNA expression of collagens 1/H9251, 1/H9251, and 3/H9251 in both nasal mucosa and polyp fibroblasts (Fig. 6A). No significant differences were found between nasal mucosa and polyp fibroblasts regarding the extent of mRNA induction by TGF-β of the three collagen types. MG262 provoked a concentration-dependent decrease in the TGF-β-induced mRNA expression of the three collagen types in both nasal mucosa and polyp fibroblasts (Fig. 6A). At 50 nM, MG262

**TABLE 1**

Effect of MG262 on the cell cycle and DNA replication of nasal fibroblasts

| Cell Treatment | G0/1 Phase, n = 6 | S Phase, n = 6 | G2/M Phase, n = 6 | DNA Replicating Cells, n = 4 |
|---------------|------------------|---------------|------------------|----------------------------|
| 0% FBS        | 92 (88.7–94.8)   | 1.9 (1.1–4.2) | 5.3 (3.4–7.1)    | 4.7 (0.8–7.7)              |
| 5% FBS        | 65.3 (50.2–76.8)**| 13.3 (8.6–16.6)**| 19.2 (13.8–34.3)***| 25.8 (17.8–37.8)**         |
| MG262 (10 nM) | 91.9 (86.5–95.3)† | 2.1 (1–3.8)†† | 4.8 (2.6–8.3)††† | 0.5 (0.2–2.6)†††           |

*, p < 0.05 and **, p < 0.01 vs. 0% FBS-treated cells.
†, p < 0.05; ††, p < 0.01; and †††, p < 0.001 vs. 5% FBS-treated cells.

Fig. 4. Effect of MG262 on nasal fibroblast cell cycle. A, fibroblasts were treated with cell medium supplemented with 5% FBS with and without the simultaneous addition of MG262 (10 nM) for 24 h. Cells were then fixed/permeabilized in 70% ethanol and stained with propidium iodide, as indicated under Materials and Methods. DNA content was then measured by FACS. Graphs show representative DNA profiles of 0% FBS-, 5% FBS-, and MG262-treated fibroblasts. B, fibroblasts were treated with 5% FBS-supplemented medium with and without the simultaneous addition of MG262 (10 nM) for 22 h. Two hours before the end of this period the nucleoside EdU was added to the cell cultures. Cells were then fixed in 70% ethanol, permeabilized with Triton X-100, and processed for the analysis of EdU incorporation into DNA by FACS, as indicated under Materials and Methods. Representative graphs of proliferating (Click-iT+) and nonproliferating cells of 0% FBS-, 5% FBS- and MG262-treated fibroblasts are shown. Data are representative of six (A) and four (B) independent experiments from different patients.
inhibited the mRNA expression of all three collagens more than the basal condition (no TGF-β). Indeed, as shown in Fig. 6B, MG262 also markedly suppressed the basal expression of collagens.

We then attempted to elucidate the mechanisms by which MG262 suppressed collagen mRNA expression in nasal fibroblasts. We have already demonstrated that MG262 increased c-Jun phosphorylation induced by 5% FBS (Fig. 3, A and B). Previous reports have shown that stimulation of the JNK/activator protein-1 pathway by phorbol 12-myristate 13-acetate or estradiol leads to the suppression of type I collagen synthesis (Silbiger et al., 1999). Based on these observations, we hypothesized that MG262 would also increase c-Jun phosphorylation induced by TGF-β/H9252, and that such an increase might partly explain the suppressive effect of MG262 on collagen expression in our cells. As shown in Fig. 6C, MG262 markedly increased c-Jun phosphorylation in both basal and TGF-β/H9252-stimulated nasal fibroblasts. To elucidate whether this induced c-Jun phosphorylation was involved in the suppression of collagen expression by MG262, we analyzed the effect of MG262 in cells with a silenced expression of c-Jun.
expression by MG262 was not abrogated, however, in c-Jun siRNA-transfected nasal fibroblasts (data not shown).

The gene expression of type I collagen induced by TGF-β is mediated primarily via the Smad signaling pathway, where, after phosphorylation by the activated type I TGF-β receptor, cytoplasmic Smad2 and Smad3 heterodimerize with Smad4 and accumulate within the nucleus, where they recruit cofactors to Smad-binding elements in the collagen gene promoter and thereby activate collagen transcription (Ghosh et al., 2002). One of the numerous proteins found to repress TGF-β-induced collagen gene expression is the nuclear hormone receptor PPAR-γ (Ghosh et al., 2009). It has recently been reported that bortezomib inhibits TGF-β-mediated target gene expression in lung fibroblasts, partly by increasing PPAR-γ abundance and activity (Mutlu et al., 2012). We expected that MG262 would similarly increase PPAR-γ protein levels in nasal fibroblasts but it failed to do so in both basal and TGF-β-stimulated nasal fibroblasts (Fig. 6C).

The tumor suppressor p53 has also been reported to be a potent repressor of TGF-β-regulated collagen gene expression in fibroblasts (Ghosh et al., 2004; Nacu et al., 2008). Proteasome inhibitors are known to up-regulate the expression of p53 in multiple myeloma cells (Hideshima et al., 2003). We therefore hypothesized that MG262 would increase p53 levels in nasal fibroblasts, and that such an increase may partly explain the suppressive effect of MG262 on collagen expression. As shown in Fig. 6C, MG262 increased p53 levels in both basal and TGF-β-stimulated nasal fibroblasts. To elucidate whether this increase in p53 was in-

**Fig. 6.** Effect of MG262 on collagen mRNA expression in nasal fibroblasts. A and B, nasal mucosa and polyp fibroblasts were incubated with cell medium without FBS and MG262 for 1 h before either the addition (A) or not (B) of TGF-β (5 ng/ml) for 24 h. Total cellular RNA was then extracted and converted (1 μg) to cDNA, and the mRNA expression of collagens 1α1, 1α2, and 3α1 was analyzed by real-time PCR and normalized to the RNA polymerase II constitutive gene. Graphs show the median (bars) and interquartile range (whiskers) of n = 7 (nasal mucosa) and n = 12 (nasal polyps) (A) and n = 5 (B) independent experiments from different patients. *p < 0.05; **p < 0.01; and ***p < 0.001 versus untreated. ††, p < 0.01 and †††, p < 0.001 versus TGF-β. C, fibroblasts were incubated with cell medium without FBS and MG262 (50 nM) for 1 h before the addition or not of TGF-β (5 ng/ml) for 24 h. Cellular lysates were then obtained, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, and membranes were processed for the analysis of p-c-Jun, PPAR-γ, p53, and β-actin by Western blot. Images are representative of three independent experiments from different patients.
volved in the suppression of collagen expression by MG262, we analyzed the capacity of MG262 to suppress collagen mRNA expression in cells whose p53 activity was inhibited by cell treatment with 1 μM of the p53 inhibitor pifithrin-α. MG262 still markedly inhibited both basal and TGF-β-stimulated collagen mRNA expression in pifithrin-α-treated fibroblasts (Supplemental Fig. 1).

**Effect of MG262 on the Production of Proinflammatory Cytokines.** To determine the anti-inflammatory potential of proteasome inhibition in nasal fibroblasts, we first investigated the effect of MG262 on the production of the proinflammatory cytokines IL-6 and IL-8, which are released in high amounts by these cells (Pujols et al., 2011). As expected, the treatment of cells with 10 ng/ml of the proinflammatory stimuli IL-1β (Fig. 7A) or TNF-α (Supplemental Fig. 2) for 4 h markedly induced IL-6 and IL-8 production in both nasal mucosa and polyp fibroblasts, compared with non-stimulated cells. MG262 concentration-dependently inhibited IL-1β-induced IL-6 and IL-8 production in both fibroblast types (Fig. 7A). MG262 also inhibited TNF-α-induced IL-6 and IL-8 production in both fibroblast types, although inhibition did not reach statistical significance for IL-6 (Supplemental Fig. 2). Under the conditions tested in this study, MG262 did not significantly alter cell viability, as determined by the XTT assay, compared with non-MG262-treated cells (data not shown).
IL-1β (10 ng/ml) + inflammatory mediators is through inhibition of NF-κB and proteasome inhibitors reduce the release of a variety of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, MCP-1, RANTES, and GM-CSF. These molecules are proinflammatory cytokines and chemokines, more specifically MCP-1, RANTES, and GM-CSF.

The main proposed mechanism by which proteasome inhibitors reduce the release of a variety of pro-inflammatory mediators is through inhibition of NF-κB (Meiners et al., 2008). Nevertheless, we first determined the intracellular signaling pathways involved in IL-1β-induced IL-6 and IL-8 release. The release of IL-6 and IL-8 induced by IL-1β was completely abrogated by the pretreatment of nasal fibroblasts with the IKK/NF-κB inhibitor BMS-345541 and inhibited to a lesser extent by the p38 and JNK MAPK inhibitors SB203580 and SP600125, respectively (Fig. 7B). Consequently, we first explored whether cytokine inhibition by MG262 involved reduced NF-κB activation. MG262 prevented the translocation of the NF-κB subunit p65 to the cell nucleus induced by both IL-1β (Fig. 7C) and TNF-α (Supplemental Fig. 3) and also prevented the degradation of phosphorylated IκB-α in IL-1β-stimulated fibroblasts (Fig. 7D). In addition, treatment of cells with MG262, under the same experimental conditions that inhibited IL-6 and IL-8 release (Fig. 7A), increased the phosphorylation of p38 and c-Jun MAPK in both basal and IL-1β-stimulated fibroblasts (Fig. 7E). Therefore, the inhibitory effect of MG262 on cytokine release does not occur through the inhibition of either c-Jun or p38 MAPK.

Finally, we determined whether the inhibitory effects of MG262 on IL-6 and IL-8 production also occurred for other proinflammatory cytokines and chemokines, more specifically MCP-1, RANTES, and GM-CSF. These molecules are secreted by nasal fibroblasts upon exposure to different proinflammatory or stressful stimuli, and they are known to participate in the recruitment and activation of monocytes, lymphocytes, or eosinophils to the inflammatory site (nasal polyp) (Xing et al., 1993; Meyer et al., 1998; Nonaka et al., 1999; Le Bellego et al., 2009). MG262 provoked a significant concentration-dependent decrease in the IL-1β-induced production of MCP-1, RANTES, and GM-CSF in nasal mucosa and polyp fibroblasts (Fig. 8).

Discussion

This is the first study to assess the effect of proteasome inhibition on the proliferation and regulation of the cell’s function in primary airway fibroblasts (in this case nasal fibroblasts) from both control subjects and patients with airway inflammatory diseases (in this case nasal polyposis and asthma).

Using the XTT assay, we first found that MG262 reduced the viability of nasal fibroblasts, especially after 48 and 72 h of treatment. Cell viability was similarly reduced by the exposure of nasal fibroblasts to bortezomib. Proteasome blockade was as effective in nasal polyp as in control nasal mucosa fibroblasts. Nasal mucosa (left) and polyp fibroblasts (right) were incubated with 10% csFBS-supplemented medium in the absence or presence of 50 and 500 nM of MG262 for 1 h before the addition of IL-1β (10 ng/ml) for 4 h, MCP-1 (top), RANTES (middle), and GM-CSF (bottom) production in supernatants was measured with Cytometric Bead Array Flex Sets by FACS. Graphs show the median (bars) and interquartile range (whiskers) of five independent experiments from different patients. †, p < 0.05 versus untreated. ††, p = 0.01 versus untreated. **, p < 0.01 versus untreated. †††, p < 0.01 versus IL-1β.
tivity to MG262-induced cell death would depend on the fibroblast origin, i.e., nose versus skin. Those authors then reported that exposure of murine lung fibroblasts to 1 μM bortezomib for 24 h similarly had no effect on cell viability (Fineschi et al., 2008), which contrasts with the inhibitory effect of MG262 on nasal fibroblast viability when used at an equivalent time and concentration.

MG262 provoked the loss of mitochondrial membrane potential in nasal fibroblasts, thus confirming that the decrease in cell viability by MG262 was caused by the induction of apoptosis. The induction of this early apoptotic event by proteasome inhibitors has been reported in cancer cells (Ling et al., 2003b; Domingo-Doménech et al., 2008) and human pulmonary fibroblasts (You and Park, 2011). Our results using the caspase inhibitor z-VAD-FMK revealed that the decrease in nasal fibroblast viability provoked by MG262 involved caspase-mediated apoptosis. Accordingly, and in agreement with studies in cancerous cells (Hideshima et al., 2001; Ling et al., 2003a,b) and noncancerous cells (Kawakami et al., 1999; Goldbaum et al., 2006), MG262 induced the activation of the apoptotic enzymes caspase-3 and PARP in nasal fibroblasts. However, compared with cancer cell lines, these enzymes seem to be much less activated by proteasome inhibition in our cells, thus requiring very high concentrations of MG262 (1000 nM) for at least 24 h to be detected. This may be because nasal fibroblasts are more resistant to proteasome inhibitor-mediated apoptosis than other cell types, or because proteasome inhibitor-mediated apoptosis in our cells primarily involves apoptotic mediators other than caspase-3 and PARP.

In line with studies reported in tumor cells (Hideshima et al., 2003; Yang et al., 2004), MG262 provoked a strong activation (phosphorylation) of c-Jun in nasal fibroblasts. The involvement of c-Jun activation in MG262-induced cell death was further demonstrated in transfection experiments with c-Jun siRNA. MG262 also activated MKP-1 expression in our cells. The induction of MKP-1 by proteasome inhibitors is reported to play an antiapoptotic role through down-regulation of JNK activity (Small et al., 2004). Effectively, the overexpression of MKP-1 in cancer is reported to be a bad prognostic factor for curing the disease (Small et al., 2007). In our fibroblasts, MG262-induced MKP-1 expression does not seem to down-regulate c-Jun activity, because the phosphorylation of c-Jun was strongly induced by MG262, but a possible relationship between these two events has not been investigated. Conversely, the current study has not explored whether inhibition of MKP-1 would further enhance the JNK pathway and, as a result, increase the apoptotic capacity of MG262.

Proteasome inhibitors cause cell-cycle arrest, particularly a G2/M arrest, in cancer cells (Ling et al., 2003a; Codony-Servat et al., 2006; Domingo-Doménech et al., 2008). The use of synchronized (growth-arrested) cultures showed that this arrest takes place not only in G1/M but also in G0, G2, or G2/S transition in nasal fibroblasts. In keeping with the blocking effects of MG262 on the cell cycle, MG262 inhibited the proliferation (DNA replication) of nasal fibroblasts. These results concur with the marked inhibition of cell proliferation after the incubation of fibroblast-like synoviocytes with bortezomib, at the same concentration (10 nM) used in our study (Yannaki et al., 2010).

MG262 inhibited Rb phosphorylation and, in agreement with data reported in cancerous cells (Hideshima et al., 2001; Ling et al., 2003a; Codony-Servat et al., 2006), increased the expression of p21 and p27 in nasal fibroblasts. Because p21 and p27 are well known proteasome substrates (Adams, 2003), their increase by MG262 is probably the result of their inhibited degradation. The increase in cyclin D1 levels after fibroblast treatment with MG262 is unsurprising, because cyclin D1 is also degraded by the proteasome (Guo et al., 2005). Therefore, the lack of Rb phosphorylation, and consequently the inhibition of cell-cycle progression, provoked by MG262 in nasal fibroblasts must be the consequence of increased p21 and p27 rather than any lack of cyclin D1.

We have explored the capacity of MG262 to down-regulate the profibrotic function of nasal fibroblasts. We reported previously that TGF-β induced the mRNA expression of collagen types 1α1, 1α2, and 3α1 in nasal fibroblasts, an induction not abrogated by glucocorticoid treatment (Pujols et al., 2011). We now report that moderately low concentrations of MG262 (10–50 nM) significantly reduce the mRNA expression of the three collagen types in both unstimulated and TGF-β-stimulated nasal mucosa and polyf fibroblasts. Likewise, a decrease in collagen mRNA expression has been reported after proteasome inhibition of human dermal fibroblasts (Fineschi et al., 2006; Goffin et al., 2010) and murine lung fibroblasts (Fineschi et al., 2008). Proteasome inhibition in rat cardiac fibroblasts also decreased collagen mRNA expression and expression of matrix metalloproteinases 2 and 9 (Meiners et al., 2004). It has recently been reported that bortezomib inhibits TGF-β-mediated target gene expression in human lung fibroblasts from normal individuals and patients with idiopathic pulmonary fibrosis and in skin fibroblasts from a patient with scleroderma (Mutlu et al., 2012).

The mechanisms by which proteasome inhibitors suppress collagen expression have not been fully elucidated. Numerous transcription factors and cofactors are known to be involved in the regulation of both basal and TGF-β-stimulated type I collagen gene expression, including, among others, SP1, activator protein-1, different Smad family members, CBP/p300, PPAR-γ, and p53 (Ghosh, 2002; Ghosh et al., 2009). As opposed to results reported in lung fibroblasts (Mutlu et al., 2012), PPAR-γ levels were not increased by exposure of nasal fibroblasts to MG262. We found that MG262 increased c-Jun phosphorylation and p53 levels in both unstimulated and TGF-β-stimulated nasal fibroblasts, but none of these effects could explain the marked suppressive effect of MG262 on collagen expression. With regard to other transcription factors involved in TGF-β-induced collagen expression, Goffin et al. (2010) reported that bortezomib did not affect TGF-β-induced Smad2 phosphorylation, nuclear translocation, or binding to the collagen 1α2 promoter. In fact, Fineschi et al. (2008) reported an increase in TGF-β-induced Smad2 phosphorylation by proteasome inhibitors. Mutlu et al. (2012) similarly found that bortezomib did not inhibit TGF-β-induced Smad3 phosphorylation or nuclear translocation, although it inhibited the transcription of a Smad-responsive luciferase reporter construct. It is worth noting that bortezomib abrogated the binding of SP1 to the collagen 1α2 promoter in both untreated and TGF-β-stimulated fibroblasts (Goffin et al., 2010). Analysis of the possible role of SP1 will be the subject of future experiments but are beyond the scope of the current article.

We have also explored the capacity of MG262 to regulate the production of proinflammatory cytokines and chemokines
by nasal fibroblasts. MG262 reduced the IL-1β- and TNFα-induced release of IL-6 and IL-8 by both nasal mucosa and polyp fibroblasts. In agreement with our results, a decrease in IL-6 production has been reported after proteasome inhibition of TNF-α-stimulated airway smooth muscle cells (Moutzouris et al., 2010), lipopolysaccharide plus phorbol 12-myristate 13-acetate-stimulated U937 monocytes (Ortiz-Lazareno et al., 2008), and multiple myeloma cell lines (Hideshima et al., 2001). Controversial results have been obtained with regard to IL-8: in line with our results, proteasome inhibition of lipopolysaccharide-stimulated macrophages has led to decreased IL-8 levels (Cuschieri et al., 2004), but increased IL-8 levels have also been reported after the proteasome inhibition of various cell types (Hipp et al., 2002; Gerber et al., 2004). MG262 also decreased the IL-1β-induced production of other proinflammatory mediators, i.e., MCP-1, RANTES, and GM-CSF, which are known to participate in the recruitment and activation of inflammatory cells on the nasal polyp site (Xing et al., 1993; Meyer et al., 1998; Nonaka et al., 1999; Le Bellego et al., 2009). The inhibition of one or more of these mediators by proteasome inhibitors has also been reported in human airway smooth muscle (Moutzouris et al., 2010) and endothelial cells (Hipp et al., 2002).

The potent anti-inflammatory effects of proteasome inhibitors have been attributed mainly to attenuated activation of the proinflammatory transcription factor NF-κB (Meiners et al., 2008). Our experiments using intracellular pathway-specific inhibitors revealed that the activation of NF-κB is critical in the induction of IL-6 and IL-8 production by IL-1β in nasal fibroblasts. Although we did not directly demonstrate this, NF-κB is also likely to be critical in the induction of MCP-1, RANTES, and GM-CSF by IL-1β in nasal fibroblasts, because the expression of these genes is also regulated by NF-κB (Barnes and Karin, 1997). It is noteworthy, and in line with previous studies (Palomella et al., 1998; Codony-Servat et al., 2006), that MG262 blocked the IL-1β-stimulated activation of NF-κB in nasal fibroblasts via two distinct mechanisms: first by preventing the degradation of the NF-κB inhibitor IκBα, and second by impairing IL-1β/TNF-α-induced translocation of NF-κB to the cell nucleus.

In summary, our study shows that the proteasome inhibitor MG262, when administered at high concentrations or for long exposure times, provokes the cell death of primary nasal mucosa and polyp fibroblasts via the induction of apoptosis, by involving the expression of cleaved caspase-3 and PARP and phosphorylation of c-Jun. Lower concentrations of MG262 block the progression of the cell cycle, by involving the inhibition of Rb phosphorylation and an increase in the cell-cycle inhibitors p21 and p27. MG262 inhibits the functional activity of nasal fibroblasts by reducing both basal and TGF-β-induced expression of collagen and down-regulating the production of IL-6, IL-8, MCP-1, RANTES, and GM-CSF induced by proinflammatory stimuli. The MG262-mediated inhibition of proinflammatory cytokine production involves the inactivation of NF-κB. In conclusion, noncytotoxic treatment with MG262 reduces the proliferative, fibrotic, and inflammatory response of nasal fibroblasts, whereas high MG262 concentrations induce apoptosis. The current study provides evidence of the effect of a proteasome inhibitor and its action mechanisms in a human in vitro cell culture model. Caution is required, however, before proposing proteasome inhibition as an alternative therapeutic strategy for reducing inflammation and fibroproliferation in airway inflammatory diseases such as chronic asthma or nasal polyposis. In this respect, the antifibrotic and anti-inflammatory effects of proteasome inhibitors should be demonstrated in other cell types and, more importantly, proteasome inhibitors should be tested in vivo in animal models of the disease, with special attention being paid to the apoptotic-inducing effects of these drugs.

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Authorship Contributions

Participated in research design: Pujols, Agell, Mullol, and Picado. Conducted experiments: Pujols, Fernández-Bertolín, Fuentes-Prado, Aloibid, and Roca-Ferrer. Performed data analysis: Pujols, Roca-Ferrer, and Agell. Wrote or contributed to the writing of the manuscript: Pujols, Agell, Mullol, and Picado.

References

Adams J (2003) The proteasome: structure, function, and role in the cell. Cancer Treat Rev 29 (Suppl 1):S3–9.
Bachert C, Van Brusene N, Toskala E, Zhang N, Olze H, Scadding G, Van Drunen CM, Mullol J, Cardell L, Gevaert F, et al. (2009) Important research questions in allergy and related diseases: 3-chronic rhinosinusitis and nasal polyposis—a GA- LEN study. Allergy 64:520–533.
Barnes PJ and Karin M (1997) Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071.
Burgess JK (2009) The role of the extracellular matrix and specific growth factors in the regulation of inflammation and remodelling in asthma. Pharmacol Ther 122:19–29.
Carmignac V, Quéré R, and Durbeej M (2011) Proteasome inhibition improves the anti-inflammatory activity in lipopolysaccharide-stimulated human bronchial epithelial cells. Am J Respir Cell Mol Biol 45:241–249.
Adams J (2003) The proteasome: structure, function, and role in the cell. Cancer Treat Rev 29 (Suppl 1):S3–9.
Bachert C, Van Brusene N, Toskala E, Zhang N, Olze H, Scadding G, Van Drunen CM, Mullol J, Cardell L, Gevaert F, et al. (2009) Important research questions in allergy and related diseases: 3-chronic rhinosinusitis and nasal polyposis—a GA- LEN study. Allergy 64:520–533.
Barnes PJ and Karin M (1997) Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071.
Burgess JK (2009) The role of the extracellular matrix and specific growth factors in the regulation of inflammation and remodelling in asthma. Pharmacol Ther 122:19–29.
Carmignac V, Quéré R, and Durbeej M (2011) Proteasome inhibition improves the anti-inflammatory activity in lipopolysaccharide-stimulated human bronchial epithelial cells. Am J Respir Cell Mol Biol 45:241–249.
Peroxisome proliferator-activated receptor-γ agonists Smad-dependent collagen stimulation by the transcriptional coactivator. *FASEB J* 23:2968–2975.

Goffin L, Seguin-Estevez Q, Alvarez M, Reith W, and Chizzolini C (2010) Transcriptional regulation of matrix metalloproteinase-1 and collagen IΑ2 explains the anti-inflammatory effect exerted by proteasome inhibition in human dermal fibroblasts. *Arthritis Res Ther* 12:R73.

Goldbaum O, Vollmer G, and Richter-Landsberg C (2006) Proteasome inhibition by MG-132 induces apoptotic cell death and mitochondrial dysfunction in cultured rat brain oligodendrocytes but not in astrocytes. *FASEB J* 20:2995–3011.

Hoyle S, Bichsel T, Zebda N, and Remacle J (2005) Peroxisome proliferator-activated receptor-γ (PPARγ) and its ligands: modulators of transcriptional activity. *Arthritis Rheum* 52:1675–88.

Hideshima T, Mitsiades C, Akiyama M, Hayashi T, Chauhan D, Richardson P, Go LH, Ghosh AK, et al. (2012) Proteasomal inhibition after injury prevents mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood* 110:1530–1534.

Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, and Anderson KC (2001) The proteasome inhibitor PS-341 induces growth inhibition, apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 61:3071–3076.

Hipp MS, Urbich C, Mayer P, Wischhusen J, Weller A, Laule M, Stangl V, Guenther C, Godes M, Mrozikiewicz A, Baumann G, and Stangl K (2004) Downregulation of matrix metalloproteinase-2 by proteasome inhibitor. *Exp Cell Res* 293:91–99.

Huang J, Brand S, Elliott PJ, et al. (1998) Role of the proteasome and NF-κB in the regulation of synovial cell apoptosis. *Cancer Lett* 124:244–248.

Le Belego F, Perera H, Plante S, Chakir J, Hamid Q, and Ludwig A (2009) Mechanical strain increases cytokine and chemokine production in bronchial fibroblasts from asthmatic patients. *Allergy* 64:32–39.

Ling YH, Liebes L, Zou Y, and Perez-Soler R (2003b) Reactive oxygen species induce expression of G0/M phase arrest and apoptosis in human non-small cell lung cancer cells. *Clin Cancer Res* 9:1145–1154.

Ling YH, Liebes L, Zou Y, and Perez-Soler R (2003b) Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. *J Biol Chem* 278:33714–33723.

Liu Y, Shepherd EG, and Nelin LD (2007) MAPK phosphatases–regulating the immune response. *Nat Rev Immunol* 7:292–312.

Ludwig A, Fechner M, Wilck N, Meiners S, Grimbo N, Baumann G, and Stangl V (2008) Proteasome inhibitors: poisons and remedies. *Med Res Rev* 28:309–327.

Meyer JE, Berner I, Teran LM, Bartels J, Sticherling M, Schröder JM, and Maune S (1998) RANTES production by cytokine-stimulated nasal fibroblasts: its inhibition by glucocorticoids. *Int Arch Allergy Immunol* 117:69–76.

Moutzouris JP, Che W, Ramsay EE, Manetsch M, Alkhouri H, Bjorkman AM, Ortiz-Larrazo PC, Hernandez-Floros G, Dominguez-Rodriguez JR, Larría-Díaz L, Palombella VJ, Conner EM, Fuseler JW, Destree A, Davis JM, Laroux FS, Wolf HE, Huang J, Brand S, Elliott PJ, et al. (1998) Role of the proteasome and NF-κB in streptococcal cell-wall induced polymyositis. *Proc Natl Acad Sci U S A* 95:13567–13571.

Pawlitzek R, Lewandowska-Polak A, and Kowalski ML (2005) Pathogenesis of nasal polyps: an update. *Curr Allergy Asthma Rep* 5:403–411.

Piffli MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

Poznic M (2009) Retinolblastoma protein: a central processing unit. *J Biocell* 34:305–312.

Pujol J, Fuentes-Prado M, Fernández-Bértoli L, Abadl I, Roca-Ferrer J, Mullol J, and Picardo M (2011) Lower sensitivity of nasal polyp fibroblasts to glucocorticoid anti-inflammatory effects. *Respir Med* 105:218–225.

Schmidt N, Gonzalez E, Visekruna A, Kuhl AA, Lobenhkenper C, Molleknopf H, Kattula SH, Steinhoff U, and Itoh N (2010) Targeting the proteasome: partial inhibition of the proteasome by bortezomib or deletion of the immunobasinub immunophilin LMPT attenuates experimental colitis. *Gut* 59:896–906.

Sherr CJ and Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1 phase progression. *Genes Dev* 13:1501–1512.

Silbiger S, Lei J, and Neugarten J (1999) Estradiol suppresses type I collagen synthesis in mesangial cells via activation of activator protein-1. *Kidney Int* 55:1258–1267.

Small GW, Shi YY, Edmund NA, Somasundaram S, Moore DT, and Orlovski RZ (2004) Evidence that mitogen-activated protein kinase phosphatase-1 induction by proteasome inhibitors plays an antiproliferative role. *Mol Pharmacol* 66:1478–1490.

Small GW, Shi YY, Higgins LS, and Orlovski RZ (2007) Mitogen-activated protein kinase phosphatase-1 is a mediator of breast cancer chemoresistance. *Cancer Res* 67:4459–4466.

Xing Z, Jordan M, Bracca T, Okhtis T, and Gaudie J (1993) Lipopolysaccharide induces expression of granulocyte/macrophage colony-stimulating factor, interleukin-8, and interleukin-6 in human nasal, but not lung, fibroblasts: evidence for heterogeneity within the respiratory tract. *Am J Respir Cell Mol Biol* 6:255–263.

Wang J and Maldonado MA (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Immunol* 244:240–248.

Wang J and Maldonado MA (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Immunol* 244:240–248.

Wang J and Maldonado MA (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Immunol* 244:240–248.

Wang J and Maldonado MA (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Immunol* 244:240–248.

Wang J and Maldonado MA (2006) The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Immunol* 244:240–248.