Prevention of Bleomycin-Induced Pulmonary Fibrosis by a Novel Antifibrotic Peptide with Relaxin-Like Activity

Alessandro Pini, Ronen Shemesh, Chrishan S. Samuel, Ross A. D. Bathgate, Arie Zauberman, Chen Hermes, Assaf Wool, Daniele Bani, and Galit Rotman

Compugen Ltd., Tel Aviv, Israel (R.S., A.Z., C.H., A.W., G.R.); Department of Anatomy, Histology, and Forensic Medicine, University of Florence, Florence, Italy (A.P., D.B.); and Howard Florey Institute and Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, Australia (C.S.S., R.A.D.B.)

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ABSTRACT

Pulmonary fibrosis is a progressive and lethal lung disease characterized by accumulation of extracellular matrix and loss of pulmonary function. No cure exists for this pathologic condition, and current treatments often fail to slow its progression or relieve its symptoms. Relaxin was previously shown to induce a matrix-degrading phenotype in human lung fibroblasts in vitro and to inhibit pulmonary fibrosis in vivo. A novel peptide that targets the relaxin RXFP1/LGR7 receptor was recently identified using our computational platform designed to predict novel G protein-coupled receptor peptide agonists. In this study, we examined the antifibrotic properties of this novel peptide, designated CGEN25009, in human cell-based assays and in a murine model of bleomycin-induced pulmonary fibrosis. Similar to relaxin, CGEN25009 was found to have an inhibitory effect on transforming growth factor-β1-induced collagen deposition in human dermal fibroblasts and to enhance MMP-2 expression. The peptide’s biological activity was also similar to relaxin in generating cellular stimulation of cAMP, cGMP, and NO in the THP-1 human cell line. In vivo, 2-week administration of CGEN25009 in a preventive or therapeutic mode (i.e., concurrently with or 7 days after bleomycin treatment, respectively) caused a significant reduction in lung inflammation and injury and ameliorated adverse airway remodeling and peribronchial fibrosis. The results of this study indicate that CGEN25009 displays antifibrotic and anti-inflammatory properties and may offer a new therapeutic option for the treatment of pulmonary fibrosis.

Introduction

Fibrosis is a pathophysiological response to chronic injury and inflammation that manifests as abnormal and excessive deposition of collagen and other extracellular matrix components. The accumulation of matrix material can disrupt the normal tissue architecture of an organ and may lead to its dysfunction (Kisseleva and Brenner, 2008; Paz and Shoenefeld, 2009). Despite the diverse etiology of various fibrotic disorders, such as cardiac fibrosis, idiopathic pulmonary fibrosis, cirrhosis of the liver, and renal fibrosis, the presence of myofibroblasts (activated collagen-secreting fibroblasts) in the affected tissue is typical of all fibrotic diseases (Wynn, 2007; Kisseleva and Brenner, 2008).

Fibroblast activation is characterized by a marked increase in the expression of type I and III collagens, fibronectin, and α-smooth muscle actin and is initiated through activation of downstream pathways by profibrotic factors such as transforming growth factor-β1 (TGF-β1) and angiotensin II (Wynn, 2008). Fibrosis occurs when the synthesis of new collagen by myofibroblasts exceeds the rate at which it is degraded, such that the total amount of collagen increases over time (Wynn, 2008). Collagen turnover and extracellular matrix remodeling is regulated by collagen-degrading enzymes—matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Uncontrolled elevation of TIMPs, which in turn causes a decrease in MMP activity, can lead to excessive collagen deposition and result in pathogenic fibrosis (Lagent et al., 2005).

Pulmonary fibrosis is the end stage of a wide range of lung diseases characterized by excessive deposition of extracellular matrix material and loss of alveolar architecture. The end result is a progressive loss of lung elasticity and function that occurs in acute interstitial pneumonia, idiopathic pulmonary fibrosis, silicosis, hypersensitivity pneumonitis, and radiation pneumonitis. As the disease advances, the pulmonary system becomes less compliant, leading to a decrease in the overall functional residual capacity of the lungs, and progressive hypoxemia. As a result, patients often require ventilator support and mechanical ventilation in order to maintain adequate oxygenation. In severe cases, the disease can result in respiratory failure and death (Wynn, 2008).

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ABBREVIATIONS: TGF-β1, transforming growth factor-β1; OD, optical density; DMEM, Dulbecco’s modified Eagle’s medium; hRLX, human H2 relaxin; MPO, myeloperoxidase; TBARS, thiobarbituric acid-reactive substances; NT, nitrotyrosine; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAS, periodic acid-Schiff; MDA, malondialdehyde; NO, nitric oxide; ANOVA, analysis of variance.
involving pulmonary fibrosis. This devastating condition leads to progressive lung destruction and scarring, characterized by the loss of alveolar structure through the apoptosis of epithelial and endothelial cells, accumulation of myofibroblasts, excessive deposition of extracellular matrix, and abnormal remodeling of lung parenchyma (Hardie et al., 2009). Pulmonary fibrosis begins with repeated injury to the lining of the alveoli, which eventually leads to extensive remodeling of the distal airspace and lung, thereby stiffening the lungs and making breathing difficult. The most common form of pulmonary fibrosis is idiopathic pulmonary fibrosis. The prognosis for patients with idiopathic pulmonary fibrosis is poor, and current therapies are ineffective at preventing or even delaying the onset of respiratory failure. Novel therapeutic approaches include molecular targeting of specific signaling pathways activated during fibrotic processes (Gharane-Kermani et al., 2009).

Relaxin, a pleiotropic hormone with known extracellular matrix remodeling capabilities, exhibits antifibrotic and anti-inflammatory activities (Masini et al., 2004; Samuel et al., 2007a). Through activation of its specific GPCR receptor, RXFP1/LGR7, relaxin acts at multiple levels to inhibit fibrogenesis (van der Westhuizen et al., 2008). This includes inhibition of inflammatory cell influx into injured organs; amelioration of the influence of profibrotic factors, primarily TGF-β1, on the acceleration of fibroblast proliferation, differentiation into myofibroblasts, subsequent myofibroblast-induced collagen deposition and overexpression; and stimulation of collagen degradation via increasing expression of various MMPs, while reducing the activity of tissue inhibitors of MMPs (TIMPs) (Samuel et al., 2007a,b). Based on these combined actions, recombinant H2 relaxin, the major stored and circulating form of human relaxin, has been shown to display beneficial antifibrotic properties in a number of animal models of induced fibrosis (Formigli et al., 2007; Samuel et al., 2007a,b).

We have recently identified a novel peptide, P74, that activated the relaxin RXFP1/LGR7 receptor in transiently transfected CHO cells (Shemesh et al., 2008). The P74 peptide, which we named CGEN25009, showed activation of cAMP in Chinese hamster ovary cells overexpressing LGR7, similar to that of H2 relaxin (Shemesh et al., 2009). In this study, we set out to further characterize the activation of the RXFP1/LGR7 receptor pathway by CGEN25009 using THP-1 cells that natively express this receptor. Furthermore, we examined the antifibrotic activity of CGEN25009 using in vitro assays of fibrosis, namely collagen deposition and MMP-2 expression in human fibroblasts, as well as an in vivo model of bleomycin-induced pulmonary fibrosis. The results presented here indicate that CGEN25009 exhibits anti-inflammatory and antifibrotic activities, similar to those of H2 relaxin, and could offer therapeutic benefits for conditions involving pulmonary fibrosis.

Materials and Methods

Peptide Synthesis. The CGEN25009 (P74) peptide is derived from the hypothetical protein Complement C1q tumor necrosis factor-related protein 8 (C1QTS_HUMAN) (Shemesh et al., 2008). The peptide was chemically synthesized by the solid-phase peptide synthesis method and purified by reversed-phase high-performance liquid chromatography (Sigma-Aldrich, Milan, Italy). The sequence of the synthetic peptide is: GQKGGVQPPGAYRRAAYAFFVGRRAAYAFFSV-Amide, in which the underlined valine residue replaced the natural cysteine residue to prevent spontaneous dimerization. The C-terminal glycine was replaced by an amide, and the N terminus remained as free amine. The peptide’s identity was verified by mass spectrometry. Final purity of the peptide was >90% as measured by reversed-phase high-performance liquid chromatography.

Cell Culture. The human monocytic cell line THP-1 was obtained from Centro Strutture cellulari—Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (Brescia, Italy). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 250 U/ml penicillin, and 250 μg/ml streptomycin. All reagents were purchased from Sigma-Aldrich. The human dermal fibroblast cell line BJ3 (Hahn et al., 1999) was kindly provided by Professor William C. Hahn (Department of Biology, Massachusetts Institute of Technology, Whitehead Institute for Biomedical Research, Cambridge, MA) and was previously shown to express RXFP1/LGR7 mRNA up to 33 passages in culture (Christian S. Samuel, unpublished data). For assessment of collagen deposition and MMP-2 activity, BJ3 cells were used between passages 10 and 15 and maintained in DMEM containing 15% FCS (DMEM-FCS).

Activation of cAMP, cGMP, and Nitric Oxide in THP-1 Cells. THP-1 cells were seeded at 4 × 10^5 cells/0.5 ml in 24-well plates and subjected to stimulation with either human H2 relaxin (hRLX) or CGEN25009 at final concentrations of 10 nM, 100 nM, and 1 μM. Recombinant hRLX was kindly supplied by Prof. Mario Bigazzi (Relaxin Foundation, Prosperus Institute, Florence, Italy). These concentrations were selected based on those shown previously to induce intracellular cAMP levels in THP-1 cells by hRLX (Bani et al., 2007). Forskolin (Sigma-Aldrich), an adenylyl cyclase activator, was used at 100 μM as a positive control for cAMP induction. Stimulation with the noted substances was maintained for 15 min concurrently with the second, durable peak of cAMP in response to relaxin (Nguyen et al., 2003), in the presence of 3-isobutyl-1-methoxyxanthine (Sigma-Aldrich) at 100 μM to inhibit phosphodiesterase activity. Each experimental point was performed in triplicate. At term incubation, the samples were centrifuged for 8 min at 3400 g, and the cell pellet was resuspended in 500 μl of ethanol 70% at 4°C and frozen at −20°C.

Measurements of intracellular cAMP and cGMP were carried out using the appropriate commercial enzyme-linked immunoabsorbent assay kits (cAMP or cGMP; Direct Biotak EIA; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer’s instructions. The values are expressed as femtomoles of cAMP or cGMP per 4 × 10^5 cells and calculated as mean ± S.E.M. Evaluation of nitric oxide (NO) production was performed by measuring the accumulation of nitrite, a stable end product of NO metabolism, in the supernatant of THP-1 cells. Nitrite levels were determined spectrophotometrically by the Griess reaction, adapted for a 96-well plate reader. In brief, 100 μl of sample were added to 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 5% phosphoric acid; Sigma-Aldrich). The optical density (OD) at a wavelength of 546 nm was measured with a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Milan, Italy). Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of NaNO2 dissolved in culture medium. The values are expressed as nanomoles of nitrite per 4 × 10^5 cells and calculated as mean ± S.E.M. Statistical comparison of the differences between the experimental values was carried out with one-way ANOVA and appropriate post hoc test, using Prism software (ver. 4.03; GraphPad Software, San Diego, CA).

Determination of MMP-2 Activity from Dermal Fibroblasts. Human dermal fibroblasts (BJ3) were used to evaluate the effect of CGEN25009 on MMP-2 stimulation, over the level induced by TGF-β1 (2 ng/ml) alone, as described previously (Samuel et al., 2004). Cells were plated at a density of 10^5 cells/well in 12-well plates, maintained in DMEM-FCS (1 ml/well) and immediately treated with TGF-β1 (2 ng/ml) plus H2 relaxin (100 ng/ml; 16.8 nM)-treated cells were used as positive control. BJ3 cells were then exposed to the same treatment groups in serum-free media containing lactalbumin hy-
drolysat for a further 24 h (because serum interferes with zymographic analysis of MMP-2). After 72 h, the conditioned media were collected and measured for protein content using the Bradford protein assay (Bio-Rad Laboratories). Samples containing an equal amount of protein were diluted 1:100 to 1:125 (to avoid saturation of the MMP-2 bands) and loaded onto gelatin zymograph gels containing 7.5% acrylamide and 1 mg/ml gelatin. Gelatin zymography was performed as described previously (Woessner, 1995; Samuel et al., 2004). Clear bands indicated gelatinolytic activity. Densitometry of the MMP-2 bands was carried out using the Calibrated Imaging Densitometer and Quantity-One software (Bio-Rad GS 710). Statistical analysis was performed by one-way ANOVA, using the Newman-Keuls post hoc test to determine whether significant differences in MMP-2 expression were detected between treatment groups.

**Determination of Collagen Deposition from Dermal Fibroblasts.** Human dermal (BJ3) fibroblasts were also used to evaluate the effect of CGEN25009 on TGF-β1-stimulated collagen deposition, which was evaluated by measuring the content of hydroxyproline, an amino acid that is specifically found in collagen. Cells were plated at a density of 10^6 cells/well in six-well plates, maintained in DMEM-supplemented with H2 relaxin (100 ng/ml; 16.8 nM). After 72 h, the media were removed from each well, and the cells were exposed to 0.1 M HCl, to keep samples stable over long periods. Densitometry was performed by a scaled-down version of the Bergman and Loxley method, as described previously (Samuel et al., 1996). Hydroxyproline values were then calculated to convert collagen content by multiplying by a factor of 6.94 (based on hydroxyproline representing 14.4% of the amino acid composition of collagen), to obtain total collagen content (micrograms) per sample. Statistical analysis was performed by one-way ANOVA, using the Newman-Keuls post hoc test to determine whether significant differences in collagen content were detected between treatment groups.

**Bleomycin-Induced Lung Fibrosis Model and Experimental Protocols.** The therapeutic potential of CGEN25009 was tested in a well established in vivo model of bleomycin-induced lung fibrosis in the mouse (Moeller et al., 2008). Eight-week-old male 35BL/6 mice weighing 25 to 30 g were purchased from Harlan (Udine, Italy). Animals were kept under standard conditions, under a 12-h light/dark cycle, with free access to water and a standard rodent diet. Animal studies were conducted at CeSAL, Centre for Laboratory Animal Housing and Experimentation, the University of Florence. The experimental protocol complied with the Declaration of Helsinki and the recommendations of the European Economic Community (86/609/CEE) on animal experimentation and was approved by the ethical committee of the University of Florence.

Bleomycin (Sigma, 0.05 IU in 50 µl of saline) or saline (50 µl) were delivered as a single intratracheal injection into mice previously anesthetized with 4% chloral hydrate (Sigma-Aldrich) and operated on to expose the trachea. The saline-injected mice (i.e., noninduced) were used to assess the establishment of pulmonary fibrosis in the bleomycin-injected mice and did not undergo further treatments. The bleomycin-induced mice were treated with CGEN25009 or with the vehicle [phosphate-buffered saline (PBS)] as negative control. CGEN25009 was given either by intraperitoneal administration or by continuous infusion, as detailed below. Most animals survived the various treatments and appeared healthy throughout the experimental period.

The day of operation and bleomycin injection was assumed as day 0 of the experiment. CGEN25009 was given in two different modes of administration, preventive and therapeutic. The preventive mode of administration began on day 0 and continued for the next 14 days (i.e., concurrently with the development of lung inflammation and fibrosis), whereas the therapeutic mode of administration began on day 7 and continued for the next 14 days (i.e., upon the establishment of postinflammatory lung fibrosis). The preventive treatment was carried out for 14 days using three different protocols in separate groups of mice: 1) twice-daily injections of 100 µg CGEN25009 i.p. in 100 µl of PBS; 2) twice-daily injections of 200 µg CGEN25009 i.p. in 100 µl PBS; and 3) continuous infusion of 12 µg/day CGEN25009 by osmotic minipumps (Alzet, Cupertino, CA; filled with 200 µg of the peptide in 100 µl of PBS) implanted subcutaneously into a dorsal pouch on day 0. The therapeutic treatment was performed by twice-daily injections of 100 µg CGEN25009 i.p. in 100 µl of PBS for 14 days. For each CGEN25009-treated group, appropriate negative controls were carried out that received the corresponding amounts of PBS alone. All intraperitoneal administrations were carried out twice daily, with the exclusion of weekends.

At the end of the administration period, the mice were euthanized by cervical dislocation, and the lungs were quickly excised. The right lungs were fixed by immersion in 4% formaldehyde in PBS, embedded in paraffin, and used for histological and immunohistochemical analyses. The left lungs were quickly frozen and stored at −80°C for biochemical assays.

**Histology and Assessment of Collagen, Nitrotyrosine, Goblet Cell Hyperplasia, and Smooth Muscle Layer Thickness.** Histological sections, 6 µm thick, were cut from the paraffin-embedded lung samples. All sections were stained or immunostained in a single session to minimize artifactual differences in the staining. Photomicrographs of the histological slides were randomly taken with a digital camera connected to a light microscope equipped with a ×40 objective. Each photomicrograph corresponded to a test area of 37,000 µm². Quantitative assessment of the stained sections was performed by computer-aided densitometry. Measurements of OD and surface area were carried out using the free-share ImageJ 1.33 image analysis program (http://rsb.info.nih.gov/ij).

For assessment of lung collagen, the sections were stained with a simplified Azan method for collagen fibers according to Smolle et al. (1996) with minor modifications, in which azocarminium and orange G were omitted to reduce parenchymal tissue background. OD measurements of the anilin blue-stained collagen fibers were carried out upon selection of an appropriate threshold to include interstitial and alveolar/capillary space backgrounds. OD measurements of the stained sections were performed by computer-aided densitometry. Measurements of OD and surface area were carried out using the free-share ImageJ 1.33 image analysis program (http://rsb.info.nih.gov/ij).

For assessment of lung collagen, the sections were stained with a simplified Azan method for collagen fibers according to Smolle et al. (1996) with minor modifications, in which azocarminium and orange G were omitted to reduce parenchymal tissue background. OD measurements of the anilin blue-stained collagen fibers were carried out upon selection of an appropriate threshold to exclude air spaces and bronchial/alveolar epithelium, according to Formigli et al. (2007). Values are means ± S.E.M. of the OD measurements (in arbitrary units) of individual mice (five images each) from the different experimental groups.

Protein tyrosine residue nitration, an index of nitrosylation of proteins by peroxynitrite generated during the inflammatory reaction, was determined by nitrotyrosine (NT) immunohistochemistry, as described previously (Masini et al., 2006). Lung tissue sections were incubated overnight with rabbit polyclonal antinitrotyrosine antisera (1:100; Millipore, Billerica, MA) at 4°C, followed by goat anti-rabbit biotin conjugated as secondary antibody (1:200; Vector Laboratories, Burlingame, CA); and avidin-biotinylated enzyme complex (1:200; Vector Laboratories). Negative controls were carried out by omitting the primary or the secondary antibodies, or by replacing the NT antisera with nonimmune rabbit serum (Sigma-Aldrich, 1:50). OD measurement of NT immunostaining was carried out upon selection of an appropriate threshold to include the immunolabeled tissue surface area. Values are reported as arbitrary units, calculated as surface area × OD × 10⁻⁶, and represent the means ± S.E.M. of the measurements of individual mice (five images each) from the different experimental groups.

For morphometry of smooth muscle layer thickness and bronchial goblet cell numbers, both key markers of airway remodeling, lung tissue sections were stained with hematoxylin and eosin or with periodic acid-Schiff (PAS) staining for mucins, respectively. Digital
photomicrographs of medium- and small-sized bronchi were taken at random. Measurements of the thickness of the bronchial smooth muscle layer were carried out on the digitized images using the above-mentioned software. PAS-stained goblet cells and total bronchial epithelial cells were counted on bronchial cross-section profiles, and the percentage of goblet cells was calculated. For both parameters, values are means ± S.E.M. of individual mice (five images each) from the different experimental groups.

**Determination of MPO Levels.** Myeloperoxidase (MPO) is a marker for leukocyte accumulation in tissues (Mullane et al., 1985). Levels of MPO were measured in frozen samples of lung tissue, which were weighed and homogenized in 10 µg/mL tissue in 0.2 M PBS, pH 6, supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mg/mL Pefabloc SC, and 2.5 µg/mL aprotinin; Sigma-Aldrich), and centrifuged at 10,000 g for 30 min at 4°C. The supernatants were collected, and MPO was measured by a specific immunonassay kit (CardioMPO; Prognostix, Cleveland, OH) according to the manufacturer's instructions. Protein concentration in the lung tissue samples was determined using the Bradford method. The results are expressed as picomoles per milligram of protein. Values are means ± S.E.M. of individual mice from different experimental groups.

**Determination of MDA/TBARS Levels.** Malondialdehyde (MDA), an end product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals, is considered a reliable marker of inflammatory tissue damage (Bani et al., 1998). MDA levels were determined by measurement of the chromogen obtained from the reaction of thiobarbituric acid-reactive substances (TBARS) with 2-thiobarbituric acid. MDA is a major representative of TBARS. Approximately 100 µg of frozen lung tissue was homogenized with 1 ml of 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4. Then 0.5 ml of 2-thiobarbituric acid [1% (w/v); Sigma] and 0.5 ml of HCl [25% (v/v) in water] were added to 0.5 ml of sample. The mixture was heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3 ml of 1-butanol (Sigma-Aldrich), and the organic phase was separated by centrifugation at 2,000 g for 10 min. The absorbance of the organic phase was read spectrophotometrically at 532-nm wavelength. Protein concentration in the lung tissue samples was determined using the Bradford method. The OD readings were converted to nanomoles of TBARS (MDA equivalents), using a standard curve of 1,1,3,3-tetramethoxypropane. Results are expressed as nanomoles of TBARS per milligram of protein. Values are means ± S.E.M. of individual mice from different experimental groups.

**Statistical Analysis.** Statistical significance of the differences between the CGEN25009-treated and PBS-treated groups was evaluated by one-way ANOVA followed by Newman-Keuls post hoc multiple comparison test or, when only two groups had to be compared, by Student’s t-test for unpaired values. Calculations were made with Prism software.

**Results**

**CGEN25009 Activates the RXFP1/LGR7 Receptor Pathway on THP-1 Cells.** Previous studies, using Chinese hamster ovary cells transiently transfected with the RXFP1/LGR7 receptor, showed activation of the relaxin RXFP1/LGR7 receptor by the CGEN25009 (P74) peptide (Shemesh et al., 2008). The signaling pathways downstream of RXFP1/LGR7 that are induced by relaxin include activation of stimulatory G protein (Gs), which in turn activates adenylyl cyclase, leading to increased intracellular cAMP levels and downstream functions (van der Westhuizen et al., 2008). To further evaluate the biological activity of CGEN25009, we used THP-1 cells that natively express the RXFP1/LGR7 receptor. H2 relaxin was previously shown to promote adenylate cyclase activation in these cells in a RXFP1/LGR7-dependent manner (Nguyen et al., 2003; Figueiredo et al., 2006). Relaxin was also shown to concentration-dependently stimulate intracellular accumulation of cAMP and cGMP in THP-1 cells as well as NO generation (Parsell et al., 1996; Baccari and Bani, 2008). We analyzed the effect of increasing concentrations of CGEN25009 on levels of these second messengers in THP-1 cells. Equimolar concentrations of hRLX were used as reference. Both hRLX and the CGEN25009 peptide induced concentration-dependent increases in cAMP, cGMP, and NO (Fig. 1). At the highest concentration tested, the cAMP levels induced by CGEN25009 were 16-fold higher than basal levels, whereas cGMP levels were induced 4-fold, and NO levels were increased by 4.5-fold. These findings further support the activation of the relaxin RXFP1/LGR7 receptor by CGEN25009, although the overall induction by CGEN25009 was lower by approximately an order of magnitude compared with equimolar concentrations of hRLX.

**Antifibrotic Activity of CGEN25009 In Vitro: Effect on Collagen Deposition and MMP-2 Expression in Human Dermal Fibroblasts.** Relaxin has potent antifibrotic actions that are mediated via the RXFP1/LGR7 receptor and have been demonstrated in various cell-based assays and animal models (Samuel et al., 2007a; van der Westhuizen et al., 2008). To evaluate the antifibrotic potential of CGEN25009, we tested its ability to activate the RXFP1/LGR7 pathway in fibroblast cells, leading to collagen degradation via the action of MMP-2 (gelatinase-A). To this end, we used the BJ3 human dermal fibroblast cell line that endogenously expresses RXFP1/LGR7. These and other fibroblasts were previously shown to respond to H2 relaxin by inhibition of collagen deposition and stimulation of MMP-2, after TGF-β1 induction (Unemori and Amento, 1990; Unemori et al., 1996; Samuel et al., 2004). TGF-β1-stimulated cells were used because CGEN25009 does not affect basal collagen turnover, as mentioned below, similarly to relaxin (Unemori and Amento, 1990; Unemori et al., 1996; Samuel et al., 2004). H2 relaxin served as positive control at 100 ng/ml (16.8 nM), a concentration previously shown to exert maximal effect in such assays (Unemori and Amento, 1990; Unemori et al., 1996).

CGEN25009 showed a concentration-dependent inhibition of collagen deposition after TGF-β1 induction (Fig. 2A). The same concentrations of CGEN25009 did not affect basal levels of collagen deposition (i.e., without TGF-β1 stimulation; data not shown). CGEN25009 displayed maximal inhibition of TGF-β1-stimulated collagen deposition at 1 µM (3.27 µg/ml), to an extent similar to that obtained with 16.8 nM H2 relaxin (−60–65% inhibition). Collagen levels in all treatment groups were significantly higher than the basal levels measured in the control cells, not induced by TGF-β1, indicating that neither CGEN25009 nor H2 relaxin was able to completely inhibit the TGF-β1-stimulated collagen deposition over 72 h in culture. Combination treatment with CGEN25009 and H2 relaxin did not further down-regulate TGF-β1-stimulated collagen deposition compared with either peptide alone (Fig. 2A), further supporting the notion that these peptides might act through the same pathway.

CGEN25009 induced stimulation of MMP-2 over and above that induced by TGF-β1 alone (Fig. 2B). Maximal stimulation of MMP-2 by CGEN25009 was reached already at the lowest concentration tested, 30 nM, and was equivalent to that
obtained with 16.8 nM H2 relaxin. The finding that MMP-2 expression was stimulated to a similar extent by most concentrations of CGEN25009 tested is consistent with our previous findings, which showed that increasing concentrations of H2 relaxin, ranging from 0.1 to 100 ng/ml (16.8 pM–16.8 nM), induced MMP-2 expression to an equivalent extent (data not shown).

**Beneficial Effects of CGEN25009 in Bleomycin-Induced Lung Injury and Fibrosis.** The therapeutic activity of CGEN25009 was evaluated in a murine model of lung injury and fibrosis. The finding that MMP-2 expression was stimulated to a similar extent by most concentrations of CGEN25009 tested is consistent with our previous findings, which showed that increasing concentrations of H2 relaxin, ranging from 0.1 to 100 ng/ml (16.8 pM–16.8 nM), induced MMP-2 expression to an equivalent extent (data not shown).
inflammation and fibrosis induced by intratracheal instillation of the profibrotic agent bleomycin. This animal model is often used to study the pathogenesis and treatment of pulmonary fibrosis (Moeller et al., 2008). Bleomycin causes inflammatory and fibrotic reactions—inflammation is prominent early on, and is triggered by induction of proinflammatory cytokines, activation of macrophages and neutrophils, and overproduction of free radicals (Moeller et al., 2008). Fibrosis develops subsequently to lung injury and is characterized by expression of the profibrotic cytokine TGF-β1, fibroblast activation and proliferation, and increased collagen deposition (Cutroneo et al., 2007). The switch between inflammation and fibrosis seems to occur around day 9 after bleomycin administration (Chaudhary et al., 2006). Relaxin was previously shown to inhibit lung fibrosis in this animal model (Unemori et al., 1996).

CGEN25009 was tested in the bleomycin-induced lung fibrosis model using male C57BL/6 mice. To evaluate its protective properties, preventive and therapeutic modes of administration were used. In the preventive mode, CGEN25009 was administered immediately after bleomycin induction either by twice-daily intraperitoneal injections at two different doses (2×20 and 2×100 μg) or by continuous infusion of 12 μg/day s.c. per mouse using osmotic minipumps. This dose was similar to that previously used for relaxin in a similar model (0.5 mg/kg/day, Unemori et al., 1996). Bleomycin-induced mice that were treated with the corresponding amounts of vehicle (PBS) served as negative controls. Animals that received saline instead of bleomycin (i.e., noninduced) were used to show the establishment of pulmonary fibrosis in the bleomycin-induced mice. The extent of pulmonary fibrosis was evaluated by deposition of collagen in the lungs at day 14 after bleomycin induction. Treatment with CGEN25009 for 2 weeks, either by continuous infusion or twice-daily intraperitoneal administration, resulted in a strong inhibition of bleomycin-induced pulmonary fibrosis, as shown by a reduction of lung collagen deposition (Fig. 3A and C), reaching levels similar to those of the noninduced mice.

Similar findings were obtained when CGEN25009 was administered in a therapeutic mode (Fig. 3B) (i.e., starting at day 7 after bleomycin induction, concurrently with the onset of postinflammation fibrosis). This timing was selected...
based on the results of preliminary experiments indicating that bleomycin-induced fibrosis was histopathologically appreciable at day 7 and increased progressively in the following weeks (data not shown). In this therapeutic mode, CGEN25009, given twice daily at 2 × 100 μg i.p., was capable of significantly reducing the degree of fibrosis compared with the PBS-treated negative controls.

Preliminary experiments also showed that intratracheal instillation of saline induced a modest increase in peribronchial collagen deposition over the 3-week experimental period (data not shown), most likely because of some degree of bronchial irritation. In some cases, CGEN25009 treatment seemed to reduce lung collagen deposition even below that observed in these saline-injected mice (Fig. 3, A and B). These findings, however, were not statistically significant.

Further analysis of the lung specimens provided insight into the anti-inflammatory effects exerted by CGEN25009. In agreement with previous reports in the literature (Chandler et al., 1983), visual examination of lung specimens from bleomycin-induced mice treated with vehicle (PBS) showed a dense lymphomonocytic inflammatory infiltrate in the perivascular, peribronchial, and interalveolar stroma (Fig. 4). These histological signs of inflammation were absent in the saline-injected (noninduced) mice and seemed to be reduced or even absent in the CGEN25009-treated mice (by qualitative assessment; see Fig. 4). These findings suggest that the observed reduction of lung fibrosis by CGEN25009 may involve an anti-inflammatory mechanism. To obtain a quantitative measure of leukocyte influx, MPO levels were assessed in the lung specimens.

The subsequent assays were aimed at studying the effects of CGEN25009 on key indexes of inflammatory lung injury. Inflammatory cells, particularly neutrophils and monocytes/macrophages, locally release proinflammatory molecules, as well as large amounts of harmful reactive oxygen species and reactive nitrogen species. The overall increase in oxidative stress is a pivotal mechanism of epithelial cell damage and airway remodeling (Barnes, 1990).

Myeloperoxidase, a typical enzyme contained in the granules of neutrophils and monocytes/macrophages (Mullane et al., 1985), is considered a reliable marker for leukocyte accumulation in the inflamed tissues. The levels of MPO measured in frozen lung tissue homogenates were very low in the saline-injected (noninduced) animals and increased significantly in the bleomycin-induced mice (Fig. 5), in agreement with the observed increase in leukocyte accumulation shown by histological analysis (Fig. 4). A significant decrease in MPO was demonstrated after preventive and therapeutic treatment with CGEN25009, at all the tested doses and routes of administration (Fig. 5, A and B).

Excess superoxide anion and nitric oxide, both released by activated leukocytes, react to form peroxynitrite, which causes membrane lipid peroxidation, DNA damage, and cell injury and demise. The extent of inflammation-induced oxidative lung tissue injury was evaluated by measuring the levels of TBARS in lung tissue homogenates. TBARS are typical end-products of lipid peroxidation, which include MDA and cognate compounds, and are considered a reliable marker of inflammatory tissue damage (Janero, 1990). Nitrosative lung tissue injury was determined by immunostaining for NT, a marker of protein tyrosine residue nitration by peroxynitrite (Raina et al., 2000).

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**Fig. 4.** Representative micrographs of hematoxylin and eosin-stained lung sections from noninduced mice (saline) and bleomycin-induced mice treated in preventive mode with CGEN25009 (100 μg i.p. twice daily) or with vehicle (PBS) as negative control. The peribronchial inflammatory infiltrate (asterisks) is dense in the bleomycin-induced mice treated with PBS, and it seems to be reduced in those treated with CGEN25009. Scale bars, 50 μm.

**Fig. 5.** MPO levels, a marker for leukocyte infiltration. The amount of MPO per milligram of tissue homogenate was determined, as described under Materials and Methods, in lung specimens of the mice described in the experiment in Fig. 3. A and B, the histograms show the levels of MPO (means ± S.E.M.), expressed as picomoles per milligram of lung tissue protein, after CGEN25009 or vehicle (PBS) administration in preventive mode (A) or therapeutic mode (B). The number of animals in each group is indicated within the histograms. ***, P < 0.001 versus bleomycin + PBS.**

**Fig. 6.** Levels of TBARS, markers of tissue injury by oxidative stress. TBARS levels were measured as described under Materials and Methods, in lung specimens of the mice described in the experiment in Fig. 3. The histograms show the levels of TBARS (means ± S.E.M.), expressed as nanomoles per milligram of lung tissue protein, after CGEN25009 or vehicle (PBS) administration in preventive mode (A) or therapeutic mode (B). The number of animals in each group is indicated within the histograms. ***, P < 0.001 versus bleomycin + PBS.**
As expected, the levels of TBARS were significantly elevated in the bleomycin-induced mice compared with the saline-injected (noninduced) mice (Fig. 6). Likewise, the extension and OD of NT immunostained lung tissue increased significantly in the bleomycin-induced mice (Fig. 7). CGEN25009 was able to significantly reduce TBARS levels at all the doses and routes of administration assayed, in both preventive and therapeutic modes of treatment (Fig. 6, A and B). Consistent with the data on TBARS, CGEN25009 was able to significantly reduce NT levels at all doses and delivery modes assayed (Fig. 7, A and B), except for the lower dose (2×20 μg i.p.) given in preventive mode, which did show a trend toward lower NT levels, but the values did not reach statistical significance (Fig. 7A).

We then evaluated bronchial remodeling by measuring key histological parameters of inflammation-induced adverse bronchial remodeling (Bai and Knight, 2005): the relative number of goblet cells and thickness of the smooth muscle layer. Goblet cells, which are situated in the airway epithelium, secrete mucins and contribute to the maintenance of a protective mucous film over the airway epithelium. The fraction of goblet cells increases in response to chronic airway insults, including oxidative stress, with a resultant increase in output of mucous (Bai and Knight, 2005). As expected, the percentage of PAS-positive goblet cells over total bronchial epithelial cells, as well as the thickness of the airway smooth muscle layer, were significantly increased in the bleomycin-induced mice compared with the saline-injected (noninduced) mice (Figs. 8 and 9, respectively). The increase in these two parameters was more prominent in mice sacrificed 2 weeks later.

Fig. 7. Levels of NT, a marker for protein nitration by peroxynitrite. NT levels were determined by immunohistochemistry and computer-aided morphometry, as described under Materials and Methods, in lung specimens of the mice described in the experiment in Fig. 3. A and B, the histograms depict NT levels, after CGEN25009 or vehicle (PBS) administration in preventive mode (A) or therapeutic mode (B). Results are shown as arbitrary OD units (means ± S.E.M.). The number of animals in each group is indicated within the histograms. **, P < 0.001 versus bleomycin + PBS. C, representative micrographs of a negative control, in which nonimmune rabbit serum was substituted for the anti-NT antiserum, and of NT-immunostained sections from noninduced mice (saline) or bleomycin-induced mice treated with CGEN25009 in preventive mode at the noted dose or with vehicle (PBS). Bars = 50 μm.
after bleomycin induction (Figs. 8A and 9A) than in mice sacrificed after 3 weeks (Figs. 8B and 9B), indicating that adverse bronchial remodeling tended to resolve after 2 weeks. Administration of CGEN25009, in preventive or therapeutic modes of treatment, was able to significantly reduce both markers of bronchial remodeling at all tested doses and forms of administration (Figs. 8 and 9). Taken together, these results indicate that CGEN25009, given either simultaneously or 7 days after bleomycin, displayed remarkable beneficial effects on inflammatory lung injury and fibrosis.

**Discussion**

The data presented in this work demonstrate the anti-inflammatory and antifibrotic properties of a novel peptide, CGEN25009, previously shown to activate the relaxin receptor, RXFP1/LGR7 (Shemesh et al., 2008, 2009). The molecular mechanisms known to be induced by relaxin on its target cells involve multiple intracellular signaling pathways downstream of RXFP1/LGR7 (reviewed in Van Der Westhuizen et al., 2007). The best characterized cellular response to relaxin involves the induction of intracellular cAMP levels (Halls et al., 2009). Studies in THP-1 cells, which constitutively express the RXFP1/LGR7 receptor, showed a biphasic cAMP response to relaxin stimulation, with an early peak at 1 to 2 min and a later peak at 10 to 20 min (Nguyen et al., 2003; Halls et al., 2006), which is dependent on the activation of RXFP1/LGR7 receptor (Figueiredo et al., 2006). Relaxin also promotes NO biosynthesis by up-regulating the expression of nitric-oxide synthase, leading to increased cellular levels of cGMP (Baccari and Bani, 2008).

Two types of human cells that endogenously express the RXFP1/LGR7 receptor, THP-1 monocytes, and the BJ3 fibroblasts, were used to further characterize the activity of CGEN25009. Given the morphological and functional differences between these cell types, we chose to evaluate the effects of CGEN25009 on different end points in these two cell lines, which have previously been used to characterize the actions of relaxin on each cell type: the second messengers cAMP, cGMP, and NO in THP-1 cells and the extracellular matrix components collagen and MMP-2 in fibroblasts.

Our findings indicate that, similarly to relaxin, CGEN25009 elicits intracellular signaling in THP-1 cells, leading to concentration-dependent increases in cAMP, cGMP, and NO (Fig. 1), thus further supporting the notion that this novel peptide activates the RXFP1/LGR7 receptor. However, CGEN25009 was approximately 10-fold less potent than relaxin in the level of induction of these second messengers, which might indicate a lower affinity to the receptor, or an alternative mode of action. Despite this difference in potency in vitro, the in vivo dose of CGEN25009 that showed efficacy in the bleomycin-induced model of pulmonary fibrosis was the same as that of relaxin, previously shown to be efficacious in a similar animal model (discussed below).

When applied to several types of fibroblast cultures, relaxin displays strong antifibrotic effects, manifested in several parameters, including down-regulation of fibroblast activity; inhibition of profibrotic cytokine (TGF-β1, angiotensin II, interleukin-1β)-induced collagen deposition and accumulation; increased MMP-induced collagen degradation; and
restructuring of collagen lattices. These effects are consistent with relaxin’s known physiological roles in matrix remodelling (Unemori and Amento, 1990; Unemori et al., 1996; Samuel et al., 2004).

The antifibrotic activity of CGEN25009 was verified using human dermal fibroblasts, which naturally express the RXFP1/LGR7 receptor. Our findings indicate that, similarly to relaxin, CGEN25009 inhibited TGF-β1 induction of collagen deposition and enhanced MMP-2 expression (over and above that induced by TGF-β1 alone) in these cells. MMP-2 is most likely not the sole mechanism involved in the collagen-inhibitory actions of CGEN25009 and, in fact, may not even be a driving factor involved (on the basis of the differential concentrations of CGEN25009 required to stimulate MMP-2 versus inhibit collagen deposition). However, we suggest that CGEN25009 may act through several mechanisms to regulate collagen turnover via RXFP1/LGR7, similarly to relaxin (reviewed in Samuel et al., 2007b and subject to further investigation), one of which includes the stimulation of MMP-2. Because the combination of CGEN25009 and relaxin did not result in further inhibition of collagen deposition compared with either treatment alone, it is suggested that these two peptides act through a similar pathway via the same receptors to mediate their antifibrotic actions on dermal fibroblasts. Furthermore, at the maximal concentration at which CGEN25009 was able to inhibit TGFβ1-induced collagen deposition, it showed no effect on basal collagen levels (data not shown), pointing to the potential safety of this peptide.

We further evaluated the beneficial properties of CGEN25009 in an animal model of pulmonary fibrosis induced by intratracheal instillation of bleomycin. CGEN25009 dramatically reduced bleomycin-induced fibrosis, manifested as collagen accumulation in the lungs. These results were obtained when CGEN25009 was administered either simultaneously (preventive mode) or 7 days after bleomycin (i.e., when postinflammatory lung fibrosis had begun) (therapeutic mode). Moreover, both modes of CGEN25009 administration caused a remarkable reduction in various aspects of inflammatory lung injury, such as leukocyte infiltration, oxidative and nitrosative lung tissue damage, and adverse bronchial remodeling. Because of insufficient amounts of lung tissue, we could not perform further molecular analyses for collagen metabolism, such as TIMP/MMP expression. However, it is conceivable that the observed reduction of lung fibrosis upon administration of CGEN25009 in both preventive and therapeutic modes results from the up-regulation of collagen turnover, in keeping with the in vitro findings on cultured fibroblasts.

Using a similar animal model of pulmonary fibrosis and the same dose (0.5 mg/kg/day), relaxin was previously shown to inhibit lung fibrosis and alveolar thickening (Unemori et al., 1996). Although the anti-inflammatory effects of relaxin were not evaluated in that specific study, relaxin was shown to exert various anti-inflammatory effects in different setups (Samuel et al., 2007a), such as inhibition of inflammatory cell influx to injured organs, including neutrophils and mast cells; reduction of oxygen free radical-mediated injury; and
reduction of various markers of inflammation (Bani et al., 1998; Masini et al., 2004).

Chronic inflammatory conditions in the lungs lead to permanent structural changes and remodeling of the airway walls, of which fibrosis is a major constituent. Fibroproliferative diseases, including pulmonary fibrosis, are a leading cause of morbidity and mortality. Despite its enormous effect on human health, there are currently no approved treatments that directly target the mechanisms of fibrosis, and no proven antifibrotic therapy has shown a clear efficacy in ameliorating the clinical course of fibrotic diseases (Wynn, 2007; Paz and Shoenfeld, 2009). Although some potential treatments and compounds are being evaluated by the Food and Drug Administration, many of which target the TGFβ1 pathway, such as pirfenidone (Paz and Shoenfeld, 2009), no treatment has been approved so far. There is thus still an unmet need for selective, potent, and safe antifibrotic drugs.

The results of the present study point to CGEN25009 as a novel, potential therapeutic agent with anti-inflammatory and antifibrotic activities. Its mechanism of action seems to be mediated through the RXFP1/LGR7 receptor, of which relaxin is the known physiological ligand. Although relaxin itself is emerging as a promising therapeutic agent (Samuel et al., 2007a), it has a complex structure that consists of two peptide chains, which makes it difficult and expensive to manufacture. In this context, CGEN25009 can have a distinct advantage over relaxin for pharmaceutical purposes, being a short, single-chain peptide suitable for chemical synthesis with a reasonable cost/yield ratio.

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References


Address correspondence to: Dr. Ronen Shemesh, Compugen Ltd., 72 Pinchas Rosen St., Tel Aviv, Israel 69512. E-mail: ronens@compugen.co.il