Prevention of bleomycin induced pulmonary fibrosis by a novel anti-fibrotic peptide with relaxin like activity

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Running Title page

Running Title: A novel, Relaxin-like anti-fibrotic peptide.

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Nonstandard abbreviations used in the paper: RLX – Relaxin, hRLX – Human Relaxin, SEM - standard error of the mean, IPF - idiopathic pulmonary fibrosis.
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 which 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 anti-fibrotic properties of this novel peptide, designated CGEN25009, in human cell based assays and in a murine model of bleomycin-induced pulmonary fibrosis. Similarly to relaxin, CGEN25009 was found to have an inhibitory effect on TGF-β1-induced collagen deposition in human dermal fibroblasts, while enhancing 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 of lung inflammation and injury, and ameliorated adverse airway remodeling and peri-bronchial fibrosis. The results of this study indicate that CGEN25009 displays anti-fibrotic 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 is manifested as abnormal and excessive deposition of collagen and other extracellular matrix (ECM) components. The accumulation of matrix material can disrupt the normal tissue architecture of an organ, and may lead to its dysfunction (Paz and Shoenfeld, 2009; Kisseleva and Brenner, 2008). In spite of 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 (Kisseleva and Brenner, 2008; Wynn, 2007).

Fibroblast activation is characterized by a marked increase in the expression of type I and type III collagens, fibronectin and α-SMA (α-smooth muscle actin), and is initiated through activation of downstream pathways by profibrotic factors such as TGF-β1 (transforming growth factor-β1) and AngII (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 ECM remodeling is regulated by collagen degrading enzymes - MMPs (matrix metalloproteinases) and their inhibitors - TIMPs (tissue inhibitors of metalloproteinases). Uncontrolled elevation of TIMPs, which in turn causes a decrease in MMPs activity, can lead to excessive collagen deposition and result in pathogenic fibrosis (Lagente et al., 2005).

Pulmonary fibrosis is the end stage of a wide range of lung inflammatory conditions. 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, stiffening the lungs and making breathing difficult. The most common form of pulmonary fibrosis is idiopathic pulmonary fibrosis (IPF). The prognosis for IPF patients is poor and current therapies are ineffective in preventing or even delaying the onset of respiratory failure. Novel therapeutic approaches include molecular targeting of specific signaling pathways activated during fibrotic processes (Gharaee-Kermani et al., 2009).

Relaxin, a pleiotropic hormone with known extracellular matrix remodeling capabilities, exhibits anti-fibrotic and anti-inflammatory activities (Samuel et al., 2007a; Masini et al., 2004). Through activation of its specific GPCR receptor, RXFP1/LGR7, relaxin acts at multiple levels to inhibit fibrogenesis (van der Westhuizen et al., 2008) including 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., 2007b; Samuel et al., 2007a). 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 (Samuel et al., 2007a; Samuel et al., 2007b; Formigli et al., 2007).

We have recently identified a novel peptide, P74, which 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 CHO 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 which 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 \textit{in-vivo} model of bleomycin-induced pulmonary fibrosis. The results presented here indicate that CGEN25009 exhibits anti-inflammatory and anti-fibrotic activities, similar to those of H2 relaxin, and could offer therapeutic benefits for conditions involving pulmonary fibrosis.
Methods

Peptide synthesis

The CGEN25009 (P74) peptide is derived from the hypothetical protein Complement C1q tumor necrosis factor-related protein 8 (C1QT8_HUMAN) (Shemesh et al., 2008). The peptide was chemically synthesized by the solid phase peptide synthesis (SPPS) method, and purified by Reverse Phase HPLC (Sigma). The sequence of the synthetic peptide is: GQKGVGPPGA\underline{Y}RRA\underline{F}VSGRRYAAFSV-A, in which the underlined valine (V) residue replaced the natural cysteine (C) residue in order 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 RP-HPLC.

Cell culture

The human monocytic cell line THP-1 was obtained from Centro Substrati cellulari - Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, IZSLER, Brescia, Italy. Cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS), 250U/ml penicillin and 250μg/ml streptomycin (all reagents purchased from Sigma-Aldrich, Milan, Italy). 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, USA) and previously shown by us to express RXFP-1/LGR7 mRNA up to 33 passages in culture (unpublished data). For assessment of collagen deposition and MMP-2 activity, BJ3 cells were used between passages 10-15, and maintained in DMEM containing 15% FCS (DMEM-FCS).

Activation of cAMP, cGMP and NO in THP-1 cells

THP-1 cells were seeded at 4x10^5 cells/0.5 ml in 24-well plates, and subjected to stimulation with either H2 Relaxin (hRLX) or CGEN25009, at final concentrations of 10nM, 100nM and 1μM. Recombinant hRLX was kindly supplied by Prof. Mario Bigazzi, the Relaxin Foundation, Prosperius Institute, Florence,
Italy. These concentrations were selected based on those previously shown to induce intracellular cAMP levels in THP-1 cells by hRLX (Bani et al., 2007). Forskolin (Sigma), an adenylyl cyclase activator, was used at 100μM as a positive control for cAMP induction. Stimulation with the noted substances was maintained for 15 minutes, concurrently with the second, durable peak of cAMP in response to RLX (Nguyen et al., 2003), in the presence of IBMX (Sigma, Milan, Italy) at 100μM to inhibit phosphodiesterase activity. Each experimental point was performed in triplicate. At term incubation, the samples were centrifuged for 8 minutes at 3400g and the cell pellet was resuspended in 500μl ethanol 70% at 4°C and frozen at -20°C.

Measurements of intracellular cAMP and cGMP were carried out using the appropriate commercial ELISA kits (cAMP or cGMP, Direct Biotrak EIA, Amersham Biosciences), according to the manufacturer’s instructions. The values are expressed as fmol cAMP or cGMP per 4x10^5 cells, and calculated as mean ± SEM. Evaluation of 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, 100μl of sample were added to 100μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphtyl] ethyldiamine in 5% phosphoric acid, Sigma). The optical density at a wavelength of 546 nm was measured with a Bio-Rad 550 microplate reader (Bio-Rad, 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 nmol nitrite per 4x10^5 cells and calculated as mean ± SEM. Statistical comparison of the differences between the experimental values was carried out with one-way ANOVA and appropriate post-hoc test, using the GraphPad Prism 4.03 software.

**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 (2ng/ml) alone, as previously described (Samuel et al., 2004). Cells were plated at a density of 1x10^5 cells/well in 12-well plates, maintained in DMEM-FCS (1ml per well) and immediately treated with TGF-β1 (2ng/ml) in the absence or presence of CGEN25009 at 30nM, 100nM,
300nM, 1μM and 3μM for 48 hours. TGF-β1 (2ng/ml) plus H2 relaxin (100ng/ml; 16.8nM)-treated cells were used as positive control. BJ3 cells were then exposed to the same treatment groups in serum-free media containing lactalbumin hydrolysate for a further 24 hours (as serum interferes with zymographic analysis of MMP-2). After 72 hours, the conditioned media was collected and measured for protein content using the Bradford protein assay (Bio-Rad). Samples containing an equal amount of protein were diluted 1:100-1:125 (to avoid saturation of the MMP-2 bands) and loaded onto gelatin zymograph gels containing 7.5% acrylamide and 1mg/ml gelatin. Gelatin zymography was performed as previously described (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 GS710). Statistical analysis was performed by one-way ANOVA, using the Neuman-Keuls post-hoc test to determine if 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 1x10^6 cells/well in 6-well plates, maintained in DMEM-FCS (1ml per well) and were either untreated (control) or immediately treated with TGF-β1 (2ng/ml) in the absence or presence of CGEN25009 at 30nM, 100nM, 300nM, 1μM and 3μM for 72 hours. TGF-β1 (2ng/ml) plus H2 relaxin (100ng/ml; 16.8nM) treatment was used as positive control. CGEN25009 at 1μM was also tested in combination with H2 relaxin (16.8nM). After 72 hours, the media was removed from each well and the cells were exposed to 0.5ml of 6M hydrochloric acid (HCl), and then hydrolysed at 110°C for 20-24 hours. During this process, the collagen triple helix, which is primarily composed of glycine-proline-hydroxyproline repeats, is broken down to individual amino acids. The hydrolysed samples were then freeze-dried down to dry weight and then reconstituted in 30μl 0.1M HCl, in order to keep samples stable over long periods. Duplicate 10μl aliquots from each sample were then assessed for hydroxyproline content, using a scaled-down version of the Bergman & Loxley method, as described before (Samuel et al., 1996). Hydroxyproline values were then converted to 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 (μg) per sample. Statistical analysis was performed by one-way ANOVA, using the Neuman-Keuls post-hoc test to determine if 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 C57BL/6 mice, weighing 25-30g, were purchased from Harlan (Udine, Italy). Animals were kept under standard conditions, under a 12-h light/12-h dark lighting cycle, with free access to water and a standard rodent diet. Animal studies were conducted at CeSAL, Centre for Laboratory Animal Housing and Experimentation, of the University of Florence, Italy. 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, Italy.

Bleomycin (Sigma, 0.05 IU in 50μl saline) or saline (50μl) were delivered as a single intra-tracheal injection into mice previously anaesthetised with 4% chloralium hydrate (Sigma) and operated to expose the trachea. The saline-injected mice (i.e. non-induced) 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 either with CGEN25009, or with the vehicle (phosphate-buffered saline, PBS) as negative control. CGEN25009 was given either by intraperitoneal (i.p.) 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 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 for the next 14 days, i.e. upon the establishment of post-inflammatory lung fibrosis. The preventive treatment was carried...
out for 14 days using 3 different protocols in separate groups of mice: i) twice daily i.p. injections of 100μg CGEN25009 in 100μl PBS; ii) twice daily i.p. injections of 20μg CGEN25009 in 100μl PBS; iii) continuous infusion of 12μg/day CGEN25009 by osmotic minipumps (Alzet 1002, Cupertino, CA; filled with 200μg of the peptide in 100μl PBS) implanted subcutaneously into a dorsal pouch on day 0. The therapeutic treatment was performed by twice daily i.p. injections of 100 μg CGEN25009 in 100μl PBS for 14 days. For each CGEN25009-treated group, appropriate negative controls were carried out which received the corresponding amounts of PBS alone. All i.p. administrations were carried out twice daily, with the exclusion of weekends.

At the end of the administration period, the mice were killed 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 the sections were stained or immunostained in a single session, in order to minimize artefactual differences in the staining. Photomicrographs of the histological slides were randomly taken with a digital camera connected to a light microscope equipped with a x40 objective. Each photomicrograph corresponded to a test area of 38,700 μm². Quantitative assessment of the stained sections was performed by computer-aided densitometry. Measurements of optical density (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 fibres was carried out upon selection of an appropriate threshold to exclude aerial air spaces and bronchial/alveolar epithelium, according to Formigli et al. (2007). Values are means ± SEM of the OD measurements (in arbitrary units) of individual mice (5 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 previously described (Masini et al., 2006). Lung tissue sections were incubated overnight with rabbit polyclonal anti-nitrotyrosine antiserum (Upstate Biotechnology, Buckingham, UK; 1:100) at 4°C, followed by goat anti-rabbit biotin-conjugated as secondary antibody (Vector Lab, Burlingame, CA, USA; 1:200) and ABC complex (Vector Lab; 1:200). Negative controls were carried out by omitting the primary or the secondary antibodies, or by replacing the anti-NT antiserum with nonimmune rabbit serum (Sigma, 1:50). OD measurement of lung 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 by the formula: surface area x OD x 10^-6, and represent the means ± SEM of the measurements of individual mice (5 images each) from the different experimental groups.

For morphometry of smooth muscle layer thickness and bronchial goblet cell numbers, both key markers of airway remodelling, 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 calculated. For both parameters, values are means ± SEM of individual mice (5 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 homogenised in 10 μl/mg tissue in 0.2M PBS pH 6, supplemented with protease inhibitors (1mM PMSF, 20 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml Pefabloc SC, 2.5 μg/ml aprotinin, Sigma) and centrifuged at 10,000g for 30 min. at 4°C. The supernatants were collected and MPO was measured by a specific immunoassay kit (CardioMPO™, PrognostiX, Cleveland, OH, USA) following the manufacturer’s instructions. Protein
concentration in the lung tissue samples was determined using the Bradford method. The results are expressed as pmol/mg of protein. Values are means ± SEM of individual mice from the 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., 1998b). 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 mg of frozen lung tissue were homogenised with 1 ml of 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4. 0.5 ml of 2-thiobarbituric acid (1% w/v, Sigma) in 0.05 M NaOH and 0.5 ml of HCl (25% w./v. in water) are 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), and the organic phase was separated by centrifugation at 2,000 x g for 10 min. The absorbance of the organic phase was read spectrophotometrically at 532 nm wave length. Protein concentration in the lung tissue samples was determined using the Bradford method. The OD readings were converted to nmol of thiobarbituric acid-reactive substances (MDA equivalents), using a standard curve of 1, 1, 3, 3-tetramethoxypropane. Results are expressed as nmol of thiobarbituric acid-reactive substances (TBARS)/mg of protein. Values are means ± SEM of individual mice from the different experimental groups.

**Statistical analysis**

Statistical significance of the differences between the CGEN25009-treated and the PBS-treated groups was evaluated by one-way ANOVA followed by Newman-Keuls post-hoc multiple comparison test or, when only 2 groups had to be compared, by Student’s t test for unpaired values. Calculations were made with Prism 4.03 statistical program (GraphPad, San Diego, CA).
Results

CGEN25009 activates the RXFP1/LGR7 receptor pathway on THP-1 cells

Previous studies, using CHO 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 which are induced by relaxin include activation of stimulatory G-protein (Gs), which in turn activate adenylyl cyclase, leading to increased intracellular cAMP levels and downstream functions (van der Westhuizen et al., 2008). In order to further evaluate the biological activity of CGEN25009 we used THP-1 cells which 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 nitric oxide (NO) generation (Parsell et al., 1996; Baccari and Bani, 2008). We analysed the effect of increasing concentrations of CGEN25009 on levels of these second messengers in THP-1 cells. Equimolar concentrations of human recombinant H2 relaxin (hRLX) were used as reference. Both hRLX and the CGEN25009 peptide induced concentration-dependent increases in cAMP, cGMP and NO (Figure 1). At the highest concentration tested, the cAMP levels induced by CGEN25009 were 16-fold higher than basal levels, while cGMP levels were induced 4 fold, and NO levels 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 about an order of magnitude compared to equimolar concentrations of hRLX.

Anti-Fibrotic activity of CGEN25009 in vitro: Effect on collagen deposition and MMP-2 expression in human dermal fibroblasts

Relaxin has potent anti-fibrotic actions which are mediated via the RXFP1/LGR7 receptor, and have been demonstrated in various cell based assays and animal models (van der Westhuizen et al., 2008; Samuel et al., 2007a). In order to evaluate the anti-fibrotic 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.
(matrix metalloproteinase-2/gelatinase-A). To this end, we used the BJ3 human dermal fibroblast cell line which 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, following TGF-β1 induction (Unemori and Amento, 1990; Unemori et al., 1996; Samuel et al., 2004). TGF-β1-stimulated cells were used since 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 100ng/ml (16.8nM), 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 following TGF-β1 induction (Figure 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 1µM (3.27µg/ml), to a similar extent as that obtained with 16.8nM H2 relaxin (~60-65% inhibition). Collagen levels in all treatment groups were still significantly higher than the basal levels measured in the control cells, not induced by TGF-β1; indicating that neither CGEN25009, nor H2 relaxin, were able to completely inhibit the TGF-β1-stimulated collagen deposition over 72 hours in culture. Combination treatment with CGEN25009 and H2 relaxin did not further down-regulate TGF-β1 stimulated collagen deposition, compared to either peptide alone (Figure 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 (Figure 2B). Maximal stimulation of MMP-2 by CGEN25009 was reached already at the lowest concentration tested, 30nM, and was equivalent to that obtained with 16.8nM H2 relaxin. The finding that MMP-2 expression was stimulated to a similar extent by most concentrations of CGEN25009 tested, are consistent with our previous findings which showed that increasing concentrations of H2 relaxin, ranging from 0.1ng/ml up to 100ng/ml (16.8pM-16.8nM), 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 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 pro-inflammatory 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 appears 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 i.p. twice daily injections at two different doses (2x20 and 2x100 μg/day), or by continuous s.c. infusion of 12μg/day per mouse using osmotic minipumps. This dose was similar to that previously used for relaxin in a similar model (0.5mg/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. non-induced) were used in order 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 two weeks, by either 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. 3 A, C), reaching levels similar to those of the non-induced mice.

Similar findings were obtained when CGEN25009 was administered in a therapeutic mode (Figure 3 B), i.e. starting at day 7 after bleomycin induction, concurrently with the onset of post-inflammation fibrosis. This timing was selected based on the results of preliminary experiments which indicated 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 i.p. at 2x100 μg/day dose, was capable of significantly reducing the degree of fibrosis as compared with the PBS-treated negative controls.

Preliminary experiments also showed that intratracheal instillation of saline induced a modest increase of peri-bronchial collagen deposition over the 3-week experimental period (data not shown), most likely due to some degree of bronchial irritation. In some cases, CGEN25009 treatment appeared to reduce lung collagen deposition even below that observed in these saline-injected mice (Fig. 3A&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 peri-vascular, peri-bronchial and inter-alveolar stroma (Figure 4). These histological signs of inflammation were absent in the saline-injected (non-induced) mice, and appeared to be reduced or even absent in the CGEN25009-treated mice (by qualitative assessment - Figure 4). These findings suggest that the observed reduction of lung fibrosis by CGEN25009 may involve an anti-inflammatory mechanism. In order to obtain a quantitative measure of the leukocytes’ influx, MPO levels were assessed in the lung specimens (see below).

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

Myeloperoxidase (MPO), 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 (non-induced) animals and increased significantly in the bleomycin-induced mice (Figure 5), in agreement with the observed increase in leukocyte accumulation shown by histological analysis (Figure 4). A significant decrease in MPO was demonstrated following preventive and therapeutic treatment with CGEN25009, at all the tested doses and routes of administration (Figure 5A&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 thiobarbituric acid-reactive substances (TBARS) in lung tissue homogenates. TBARS are typical end-products of lipid peroxidation, which include malondialdehyde (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 nitrotyrosine (NT), a marker of protein tyrosine residue nitration by peroxynitrite (Raina et al., 2000).

As expected, the levels of TBARS were significantly elevated in the bleomycin-induced mice as compared to the saline-injected (non-induced) mice (Figure 6). Similarly, 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, both in preventive and therapeutic modes of treatment (Fig. 6A&B). Consistent with the data on TBARS, CGEN25009 was able to significantly reduce NT levels at all the doses and delivery modes assayed (Fig. 7A&B), except for the lower i.p. dose (2x20 μg) given in preventive mode, which did show a trend towards lower NT levels but the values did not reach statistical significance (Fig. 7A).

We then evaluated bronchial remodelling by measuring key histological parameters of inflammation-induced adverse bronchial remodelling (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 increase in response to chronic airway insults, including oxidative stress, with a resultant increase in output of mucus (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 as compared with the saline-injected (non-induced) mice (Figure 8 and 9, respectively). The increase in these two parameters was more prominent in mice sacrificed 2 weeks after bleomycin induction (Figure 8A and 9A) than in mice sacrificed after 3 weeks (Figure 8B and 9B), indicating that adverse bronchial remodelling 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 remodelling at all tested doses and forms of administration (Figures 8 and 9).

Taken together, the above 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 demonstrates the anti-inflammatory and anti-fibrotic properties of a novel peptide, CGEN25009, previously shown to activate the relaxin receptor, RXFP1/LGR7 (Shemesh et al., 2008; Shemesh et al., 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 1–2 min and a later peak at 10–20 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 nitric oxide (NO) biosynthesis by up-regulating the expression of nitric oxide synthase (NOS), leading to increased cellular levels of cGMP (Reviewed in Baccari and Bani, 2008).

Two types of human cells that endogenously express the RXFP1/LGR7 receptor, the THP-1 monocytic cells 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 characterise 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 signalling 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 about 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. In spite of this difference in potency in vitro, the in vivo dose of CGEN25009 which 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 anti-fibrotic effects, manifested in several parameters, including downregulation of fibroblast activity; inhibition of pro-fibrotic 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 remodeling (Unemori and Amento, 1990; Unemori et al., 1996; Samuel et al., 2004).

The anti-fibrotic 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 (based on the differential concentrations of CGEN25009 required to stimulate MMP-2 vs. 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. As the combination of CGEN25009 and relaxin did not result in further inhibition of collagen deposition, compared to either treatment alone, it is suggested that these two peptides act through a similar pathway via the same receptors to mediate their anti-fibrotic 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, e.g.
when post-inflammatory lung fibrosis had began (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 remodelling. Due to lack of sufficient 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 turn-over, in keeping with the in vitro findings on cultured fibroblasts.

Using a similar animal model of pulmonary fibrosis and the same dose (0.5mg/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 cells influx to injured organs, including neutrophils and mast cells, reduction of oxygen free radical-mediated injury and of various markers of inflammation (Masini et al., 2004; Bani et al., 1998b).

Chronic inflammatory conditions in the lungs lead to permanent structural changes and remodelling of the airway walls, of which fibrosis is a major constituent. Fibro-proliferative diseases, including pulmonary fibrosis, are a leading cause of morbidity and mortality. Despite its enormous impact 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 currently being evaluated by the FDA, 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 at CGEN25009 as a novel, potential therapeutic agent with anti-inflammatory and anti-fibrotic activities. Its mechanism of action appears to be mediated through the RXFP1/LGR7 receptor, of which relaxin is the known physiological ligand. While relaxin itself is emerging as a promising therapeutic agent (Samuel et al., 2007a), it has a complex structure composed of two peptide chains that 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


Footnotes:

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Legends for Figures

Figure 1: Induction of cAMP (A), cGMP (B) and NO (C) by CGEN25009 and human H2 relaxin (hRLX) in human monocytic THP-1 cells. Cells were stimulated with hRLX or CGEN25009 for 15 min., in the presence of IBMX (100 μM) to inhibit phosphodiesterase activity. Cells with no stimuli provided the basal levels. For cAMP measurements, forskolin (100 μM) served as a positive control. Intracellular levels of cAMP and cGMP, and accumulation of nitrite in the supernatants of THP-1 cells (nitrite, NO2, a stable end product of NO metabolism), were determined as described in Materials and Methods. Values represent mean ± SEM of triplicate experiments. **p<0.01; ***p<0.001 vs. basal levels.

Figure 2: Inhibition of collagen deposition (A) and stimulation of MMP-2 (B) by CGEN25009 in human dermal fibroblasts. Cells were stimulated with TGF-β1 (2 ng/ml) for 72h in the absence or presence of CGEN25009 (P74), at concentrations ranging from 30 nM up to 3 μM (see Materials and Methods). H2 relaxin (RLX, 16.8 nM) was used as positive control. A. For measurement of collagen deposition the cells were assessed for hydroxyproline content, as described in Materials and Methods. Cells not stimulated with TGF-β1 (Control) indicate basal levels of collagen. The combination of 1µM CGEN25009 and 16.8 nM H2 Relaxin was also tested. *P<0.05 vs control group; #P<0.05 vs. TGF-β1 alone. B For assessment of MMP-2 stimulation, the media was measured for protein content, and analyzed for MMP-2 activity by gelatin zymography, as described in Materials and Methods. *P<0.05 vs. TGF-β1 alone. Values represent mean ± SEM of three separate experiments, each performed in triplicate.

Figure 3: Inhibition of bleomycin-induced lung fibrosis by CGEN25009. Male C57/BL6 mice were injected intratracheally with bleomycin to induce pulmonary fibrosis, as described in Materials and Methods. Animals that received saline instead of bleomycin (i.e. non-induced) were only used in order to assess the establishment of pulmonary fibrosis in the bleomycin-injected mice. CGEN25009 was administered for 14 days either in a preventive mode, e.g. concurrently with bleomycin induction, by either IP administration or continuous infusion at the noted doses (A), or in a therapeutic mode, e.g. 7 days after bleomycin induction (B). Bleomycin-induced mice that were treated with the corresponding amounts of vehicle (PBS), served as negative controls. The amount of lung collagen was evaluated using a modified Azan staining for collagen
and quantified by computer-aided densitometry, as described in Materials and Methods. Results are shown as arbitrary optical density (OD) units (means ± SEM). The number of animals in each group is indicated within the histograms. #P<0.001 vs. saline; **P<0.01; ***P<0.001 vs. bleomycin+PBS. C, representative micrographs of modified Azan-stained lung sections taken from non-induced mice (saline) and bleomycin-induced mice treated in preventive mode with CGEN25009 (20 or 100 µg i.p. twice daily) or with vehicle (PBS), as negative control. Bars = 200 µm.

Figure 4: Representative micrographs of hematoxylin & eosin-stained lung sections from non-induced 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 peri-bronchial inflammatory infiltrate (asterisks) is dense in the bleomycin-induced mice treated with PBS, and appears to be reduced in those treated with CGEN25009. Bars = 50 µm.

Figure 5: MPO (myeloperoxidase) levels, a marker for leukocyte infiltration. The amount of MPO per mg of tissue homogenate was determined, as described in Materials and Methods, in lung specimens of the mice described in the experiment in Figure 3. The histograms show the levels of MPO (means ± SEM), expressed as pmoles per mg of lung tissue protein, following 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 vs. bleomycin+PBS.

Figure 6: Levels of thiobarbituric acid-reactive substances (TBARS), markers of tissue injury by oxidative stress. TBARS levels were measured as described in Materials and Methods, in lung specimens of the mice described in the experiment in Figure 3. The histograms show the levels of TBARS (means ± SEM), expressed as nmoles per mg of lung tissue protein, following 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 vs. bleomycin+PBS.

Figure 7: Levels of nitrotyrosine (NT), a marker for protein nitration by peroxynitrite. NT levels were determined by immunohistochemistry and computer-aided morphometry, as described in Materials and Methods, in lung specimens of the mice described in the experiment in Figure 3. The histograms depict NT
levels, following CGEN25009 or vehicle (PBS) administration in preventive mode (A), or therapeutic mode (B). Results are shown as arbitrary optical density (OD) units (means ± SEM). The number of animals in each group is indicated within the histograms. ***P<0.001 vs. bleomycin+PBS. (C) Representative micrographs of a negative control, in which non-immune rabbit serum was substituted for the anti-NT antiserum, and of NT-immunostained sections from non-induced mice (saline) or bleomycin-induced mice treated with CGEN25009 in preventive mode at the noted dose or with vehicle (PBS). Bars = 50 µm.

**Figure 8:** Goblet cell hyperplasia, a marker for bronchial remodeling. The fraction of goblet cells was determined by PAS staining and computer-aided morphometry, as described in Materials and Methods, in lung specimens of the mice described in the experiment in Figure 3. The histograms show the percent of goblet cells over total epithelial cells (means ± SEM), following 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<0.05 and **p<0.01 vs. bleomycin+PBS. C, representative micrographs of PAS-stained sections from non-induced mice (saline) or bleomycin-induced mice treated with CGEN25009 in preventive mode at the noted dose or vehicle (PBS). Arrows point at goblet cells. Bars = 50 µm.

**Figure 9:** Bronchial smooth muscle layer thickness, a histological parameter of bronchial remodeling. The smooth muscle thickness was assessed by computer-aided morphometry on hematoxylin & eosin-stained lung sections, as described in Materials and Methods, in lung specimens of the mice described in the experiment in Figure 3. The histograms show the thickness of the smooth muscle layer (means ± SEM), following CGEN25009 administration in preventive mode (A), or therapeutic mode (B). The number of animals in each group is indicated within the histograms. **P<0.01 and ***p<0.001 vs. bleomycin+PBS. (C) Representative micrographs of the sections from non-induced mice (saline), or bleomycin-induced mice treated with CGEN25009 in preventive mode at the noted dose or with vehicle (PBS). The thickness of the smooth muscle layer is indicated by double arrows. Bars = 50 µm.
Figure 2

(A) Relative collagen content (μg) across different treatments:
- Control
- No. addition
- hRLX-16.8 nM
- 30 nM
- 100 nM
- 300 nM
- 1 μM
- 3 μM
- 1 μM + hRLX-16.8 nM

(B) Relative OD MMP-2:
- Control
- No. addition
- hRLX-16.8 nM
- 30 nM
- 100 nM
- 300 nM
- 1 μM
- 3 μM

Significance levels indicated:
* p < 0.05
# p < 0.01

CGEN25009
TGF-β1
Figure 6

A

- nmol/mg protein

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B

- nmol/mg protein

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saline (n=1)
Figure A: Bar graphs showing the length (μm) of different treatment groups. The x-axis represents the treatment groups: saline, Bleomycin+PBS, 2x20 μg i.p., 2x100 μg i.p., 12.5 μg/day s.c., and Bleomycin + CGEN25009. The y-axis represents the length (μm) ranging from 0 to 50. Each group has a sample size indicated below the bar. The bars are labeled with asterisks indicating statistical significance: *** for p < 0.001.

Figure B: Bar graph showing the length (μm) of groups treated with Bleomycin+PBS and Bleomycin+CGEN25009. The x-axis represents the treatment groups: saline (n=1), Bleomycin+PBS, and Bleomycin+CGEN25009. The y-axis represents the length (μm) ranging from 0 to 40. The bars are labeled with asterisks indicating statistical significance: ** for p < 0.01.

Figure C: Micrographs showing the effects of different treatments. The images are labeled: saline, bleomycin + PBS, and bleomycin + CGEN25009 2x100 μg i.p. Arrows indicate the area of interest. The scale bars are provided for reference.