Immunomodulatory and Anti-inflammatory Activity in Vitro and in Vivo of a Novel Antimicrobial Candidate*

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The synthetic antimicrobial peptide SET-M33 has strong activity against bacterial infections caused by Gram-negative bacteria. It is currently in preclinical development as a new drug to treat lung infections caused by Gram-negative bacteria. Here we report its strong anti-inflammatory activity in terms of reduced expression of a number of cytokines, enzymes, and signal transduction factors involved in inflammation triggered by LPS from Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli. Sixteen cytokines and other major agents involved in inflammation were analyzed in macrophages and bronchial cells after stimulation with LPS and incubation with SET-M33. The bronchial cells were obtained from a cystic fibrosis patient. A number of these proteins showed up to 100% reduction in expression as measured by RT-PCR, Western blotting, or Luminex technology. LPS neutralization was also demonstrated in vivo by challenging bronchoalveolar lavage of SET-M33-treated mice with LPS, which led to a sharp reduction in TNF-α with respect to non-SET-M33-treated animals. We also describe a strong activity of SET-M33 in stimulating cell migration of keratinocytes in wound healing experiments in vitro, demonstrating a powerful immunomodulatory action generally characteristic of molecules taking part in innate immunity.

Antimicrobial peptides (AMPs),3 also known as host defense peptides, are new breakthrough molecules for the treatment of bacterial infections (1, 2). Several AMPs of natural and synthetic origin are currently being developed preclinically or clinically as new therapeutic agents for systemic, lung, and wound infections (3, 4). The growing interest in such molecules is due to the increase in bacterial multidrug resistance to traditional antibiotics and associated expectations of imminent increased medical need. Contrary to traditional antibiotics, which only kill bacterial cells or inhibit their growth, AMPs show a double mechanism that includes strong antibacterial activity with potent killing methods involving rapid membrane disruption (5) and immunomodulatory properties via activation of pro-inflammatory responses, chemoattraction, cell differentiation, activation of innate and adaptive compartments, wound healing, and apoptosis (6). All of these activities render AMPs simultaneously antibacterial and anti-inflammatory. Lung infections such as those encountered in cystic fibrosis (CF) patients, where bacteria and inflammation triggered by the infection play a major role in pathogenesis, are ideal targets for such molecules.

We isolated, optimized, and synthesized a peptide (SET-M33) that shows strong antimicrobial activity in vitro and in vivo against major Gram-negative pathogens (7–10). SET-M33 was synthesized in a branched form (11) for better stability in biological fluids (12–16). Its mode of action is based on a two-step mechanism: high affinity binding to LPS (8) and disruption of bacterial membranes. We characterized SET-M33 biological activity against a number of Gram-negative multidrug-resistant clinical isolates, including many CF isolates (9), as well as its interactions with membranes, LPS, and DNA; its in vitro toxicity against several eukaryotic cell lines (17); and its hemolytic activity, lack of immunogenicity (8), and efficacy in eradicating biofilms (18). SET-M33 production includes a procedure for the elimination of by-products suitable for industrial development of a peptide-based drug (17).

Preclinical development of SET-M33, currently underway, has already provided information about its therapeutic power in vivo in cases of sepsis, models of skin and lung infections, gene toxicity, acute toxicity in vivo, its propensity to select bacterial resistances, pharmacokinetics, and biodistribution (10). Because SET-M33 is a drug candidate for lung infections in CF, its potential as an anti-inflammatory agent is also of great interest. Here we analyzed its capacity to neutralize LPS, thus reducing gene and protein expression of the principal cytokines involved in inflammation triggered by LPS from Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli, major pathogens of lung infections, including those afflicting CF patients. Sixteen cytokines and other major factors involved in inflammation caused by bacterial LPS were analyzed in murine macrophages and human bronchial cells from a CF patient.
TNF-α expression was also evaluated in vivo after nebulization of LPS and SET-M33 in mouse lungs. Because bacterial infections are the most significant complication encountered in wound management (19), we also evaluated the performance of SET-M33 in stimulating cell migration of keratinocytes in wound healing experiments.

Results

Gene Expression of Pro-inflammatory Factors in Macrophages—SET-M33 was tested for LPS neutralization in macrophages and, consequently, for its inhibitory effects on the inflammatory cytokines TNF-α, IL1-β, MIP1, MIP2, IL6, COX2, KC, IP10, and MCP1, on iNOS, a key enzyme in the generation of nitric oxide, and on COX-2, the enzyme responsible for formation of prostanoids. Gene expression analysis by RT-PCR showed that stimulation of RAW264.7 murine macrophages with LPS from K. pneumoniae, P. aeruginosa, or E. coli induced an increase in gene expression of all proteins tested. In cells stimulated with LPS at appropriate concentrations (20 ng/ml LPS from K. pneumoniae and E. coli, 50 ng/ml LPS from P. aeruginosa, and 100 ng/ml LPS from E. coli for IL1-β) and then treated with SET-M33 (1 or 10 μM as indicated in Fig. 1), expression of pro-inflammatory cytokines and enzymes was strongly inhibited (Fig. 1A), with the exception of MIP2 induced by LPS from K. pneumoniae. Fig. 1B shows the gene expression percentage after treatment with SET-M33 with respect to the signal obtained from untreated LPS-stimulated cells minus the basal level. SET-M33 inhibited gene expression as follows: 100% for KC and MCP1 in cells stimulated with LPS from K. pneumoniae and for KC and IP10 in cells stimulated with LPS from E. coli; >90% for IL6 in cells stimulated with LPS from all bacteria, for GM-CSF in cells incubated with LPS from K. pneumoniae, for IP10 in cells stimulated with LPS from K. pneumoniae and E. coli to study inhibition of GM-CSF, KC, IP10, and MCP1. In all other cases, SET-M33 was used at 10 μM.

NF-κB Inhibition—To determine the inhibitory effect of SET-M33 on production of NF-κB, an essential intracellular factor produced by macrophages in response to stimulation by LPS, protein expression was analyzed in RAW264.7 macrophages after LPS stimulation and treatment with SET-M33. Western blotting with antibodies specific for NF-κB showed
that the peptide completely inhibited NF-κB expression in cells stimulated with 25 ng/ml LPS from *K. pneumoniae* (Fig. 2A), and immunofluorescence confirmed the disappearance of the NF-κB signal in cells stimulated with 20 ng/ml LPS from *P. aeruginosa* (Fig. 2B).

**Inhibition of Inflammatory Cytokines in Cystic Fibrosis Cells**—With a view to possible application of SET-M33 in CF lung infections, we analyzed the inhibition of inflammatory cytokines in IB3–1 bronchial cells isolated from a CF patient (20). The effects of SET-M33 on cytokine production in CF cells were evaluated by Luminex technology (21), detecting and quantifying a panel of proteins excreted into the medium. Cells were incubated for 24 h with LPS from *P. aeruginosa* or with LPS and SET-M33 at the concentration indicated in the legend of Fig. 3. Cells treated with SET-M33 showed the following inhibition with respect to cells incubated with LPS alone: IL6 and IL8, >20%; G-CSF, >14%; VEGF, >12%; IL12, >6%; RANTES, 8% (Fig. 3).

**In Vivo Neutralization of LPS**—To confirm the LPS neutralization assays obtained *in vitro*, we set up an animal model in which mice were challenged by intratracheal nebulization of LPS from *P. aeruginosa* with or without SET-M33. Nebulization was performed with a Penn Century device following the instructions of the manufacturer. Animals were sacrificed 8 h after the challenge, and bronchoalveolar lavage was collected for TNF-α measurement. Fig. 4 shows that animals treated with LPS and SET-M33 (5 mg/kg) produced about 99% less TNF-α than animals challenged with LPS only. SET-M33 alone did not stimulate any TNF-α production.

**In Vitro Cell Migration Assay**—Many natural antimicrobial peptides are known to promote cell migration, thus contributing to the healing of tissue damage such as that caused by *P. aeruginosa* to lung epithelia (22). To evaluate the ability of SET-M33 to promote cell migration, an *in vitro* assay was performed with keratinocytes (HaCaT) cultured in wells where silicon spacers were used to create a gap in the cell monolayer. SET-M33 1 μM promoted closure of the void area within 24 h, whereas only 15% of the void contained cells in untreated cul-

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**FIGURE 2.** NF-κB inhibition in macrophages. A, Western blotting showing protein expression in cells stimulated with 25 ng/ml LPS from *K. pneumoniae* (center) and treated with 10 μM SET-M33 (right). The control is the basal level in cells not stimulated with LPS (left). The peptide restored the NF-κB signal as in the control band. The presence of two bands is due to the heterodimeric form of NF-κB. β-actin was used as an experimental control (bottom panel). B, immunofluorescence showing NF-κB expression (green signal) in cells stimulated with 20 ng/ml LPS from *P. aeruginosa* (center panel) and treated with 20 μM SET-M33 (right panel) as described under “Experimental Procedures.” The control consisted of cells not stimulated with LPS (left panel). The membranes were stained with Lectin-Atto 647 (red). The green signal was abolished in cells treated with SET-M33.

**FIGURE 3.** Bio-plex analysis of cytokines expressed in IB3–1 cells induced with LPS from *P. aeruginosa* and treated with SET-M33. Data are mean ± S.D. Protein expression is reported in relative expression units (fold induction) on the y axis. IL-6, IL-8, IL-12, and G-CSF were stimulated with 20 ng/ml LPS and treated with 1 μM SET-M33. VEGF was stimulated with 20 ng/ml LPS and treated with 10 μM SET-M33. RANTES was stimulated with 40 ng/ml LPS and treated with 1 μM SET-M33. Basal levels of cytokine expression were as follows: 130.8 pg/ml for IL-6, 114.8 pg/ml for IL-8, 35.8 pg/ml for IL-12, 16.3 pg/ml for G-CSF, 365 pg/ml for VEGF, and 169.7 for RANTES. *, p < 0.05; ** p < 0.01.
The kines (TNF-α, IP10, and MCP-1) and growth and stimulating factors (VEGF and G-CSF, respectively), two enzymes responsible for the production of inflammatory mediators (COX-2 and iNOS), and the major intracellular signal transduction agent (NF-κB) involved in the cell response to LPS. All of these inflammatory agents tend to lead to selection of resistant bacteria. AMPs have a positive net charge that allows them to interact selectively with anionic bacterial membranes and other negatively charged structures, such as LPS. This binding to LPS not only activates a mechanism by which AMPs kill bacteria but, in some cases, also neutralizes LPS, promoting a sort of anti-inflammatory activity. AMPs can therefore be considered simultaneously antibacterial and anti-inflammatory, making them candidate drugs for diseases such as CF in which bacterial infections and inflammation together play a crucial role in disease progression.

SET-M33 has already been reported for its efficacy in killing bacterial pathogens in vitro and in vivo (10). Here we report its in vitro and in vivo activity in modulating cell response to inflammatory stimuli (LPS) from different bacteria of clinical and not cell division, as demonstrated by a cell proliferation assay in which cells were incubated with increasing concentrations of SET-M33 (data not shown).

Discussion

AMPs are considered one of the rare options to use as an alternative or in combination with traditional antibiotics that tend to lead to selection of resistant bacteria. AMPs have a positive net charge that allows them to interact selectively with anionic bacterial membranes and other negatively charged structures, such as LPS. This binding to LPS not only activates a mechanism by which AMPs kill bacteria but, in some cases, also neutralizes LPS, promoting a sort of anti-inflammatory activity. AMPs can therefore be considered simultaneously antibacterial and anti-inflammatory, making them candidate drugs for diseases such as CF in which bacterial infections and inflammation together play a crucial role in disease progression.

SET-M33 has already been reported for its efficacy in killing bacterial pathogens in vitro and in vivo (10). Here we report its in vitro and in vivo activity in modulating cell response to inflammatory stimuli (LPS) from different bacteria of clinical interest and its capacity to promote cell migration.

SET-M33 is already known to abate TNF-α production in vitro in cells stimulated with LPS from P. aeruginosa and K. pneumoniae (9), but nothing was published regarding its inhibitory effect on other cytokines and enzymes that together establish inflammatory processes triggered by bacterial infection. We therefore analyzed the effect of SET-M33 on 13 secreted proteins involved in inflammation, including cytokines (TNF-α, IL1-β, IL6, IL8, IL12, MIP1, MIP2, RANTES, KC, IP10, and MCP-1) and growth and stimulating factors (VEGF and G-CSF, respectively), two enzymes responsible for the production of inflammatory mediators (COX-2 and iNOS), and the major intracellular signal transduction agent (NF-κB) involved in the cell response to LPS. All of these inflammatory agents proved to be inhibited by SET-M33 when cells were treated with the peptide after stimulation with LPS from P. aeruginosa, K. pneumoniae, or E. coli. Different experimental techniques, including RT-PCR, Western blotting, immunofluorescence, and Luminex technology, were used, all of them confirming that SET-M33 was able to neutralize LPS.

SET-M33 was also analyzed for its wound repair activity in cultured cells. The results showed that cells treated with SET-M33 promoted wound closure more rapidly than untreated cells.

Lung hyperinflammation, characterized by increased production of various cytokines and agents, including those analyzed in this study, along with a large number of immune cells (23), is recognized as a key factor in CF lung destruction. Inflammation and hyperinflammation of CF lung tissue are principally caused by the basic genetic defect but aggravated by the presence of LPS released by dead bacteria or retained in live bacterial cells. The use of anti-inflammatory drugs such as ibuprofen has proven effective in slowing the progression of lung disease. Unfortunately, the high doses needed to obtain a beneficial outcome are associated with side effects, limiting its use among CF patients (24). There is clearly a need to find new therapies for controlling hyperinflammation in CF.

SET-M33 greatly reduced TNF-α production in vivo when LPS from P. aeruginosa and the peptide were nebulized directly into mouse lungs. The plan is to administer SET-M33 to CF patients by aerosol. After demonstration of its bactericidal activity in vitro by intratracheal inoculation (10), the demonstration of a reduction in major inflammatory cytokines in vivo is of great significance for the treatment of CF patients with this molecule.

SET-M33 is currently in preclinical development as a new aerosol drug for resistant lung infections in CF patients. Its potential as a strong LPS inhibitor makes this molecule a candidate for treating the most severe cases of lung damage in CF.

Experimental Procedures

Peptide Synthesis

SET-M33 was produced in tetrabranch form by solid-phase synthesis using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on Fmoc4-Lys2-Lys-b-Ala Wang resin with a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany). Side chain-protecting groups were 2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg, t-butoxycarbonyl for Lys, and t-butyl for Ser. The final product was cleaved from the solid support, deprotected by treatment with TFA containing tri-isopropylsilane and water (95/2.5/2.5), and precipitated with diethyl ether. Crude peptide was purified by reverse-phase chromatography on a column for medium-scale preparation in linear gradient form for 30 min using 0.1% TFA/water as eluent A and methanol as eluent B. The purified peptide was obtained as trifluoroacetate salts (TFacetate). Exchange from TFacetate (toxic by-product) to acetate form was carried out using a quaternary ammonium resin in acetate form (AG1-X8, 100–200 mesh, 1.2 meq/ml capacity). The resin-to-peptide ratio was 2000:1. Resin and peptide were stirred for 1 h, the resin was filtered off and washed exen-
sively, and the peptide was recovered and freeze-dried (17). Final peptide purity and identity were confirmed by reverse-phase chromatography on a Phenomenex Jupiter C18 analytical column (300 Å, 5 mm, 250×4.6 mm) and by mass spectrometry MALDI-TOF/TOF.

Cell Cultures

RAW264.7 murine macrophages and HaCaT human keratinocytes, purchased from Istituto Zooprofilattico Sperimentale (Brescia, Italy), were grown in their recommended medium, DMEM supplemented with 10% fetal calf serum, 200 μg/ml glutamine, 100 μg/ml streptomycin, and 60 μg/ml penicillin, and maintained at 37 °C under 5% CO2. IB3–1 cells (ATCC), derived from a CF patient with a F508/W1282X mutant genotype and immortalized with adeno12/SV40, were grown in LHC-8 supplemented with 5% FBS in the absence of gentamycin at 37 °C under 5% CO2 (20).

In Vitro Anti-inflammatory Activity

Gene Expression—RAW264.7 cells were seeded in 6-well plates (5 × 105 cells/well) and cultured overnight in a CO2 incubator. They were stimulated with 50 ng/ml LPS from P. aeruginosa (serotype 10, strain ATCC 27316, Sigma-Aldrich, L 9143), 20 ng/ml LPS from K. pneumoniae (strain ATCC 15380, Sigma-Aldrich, L 4268), or 20 ng/ml LPS from E. coli (026:B6, Sigma-Aldrich, L 8274) in the presence of 10 or 1 μM SET-M33 in DMEM for 6 h at 37 °C. Total RNA was extracted using an RNeasy kit (Qiagen, Germantown, MD) according to the instructions of the manufacturer. One-step RT-PCR (Qiagen) was applied for retrotranscription and mouse cDNA amplification of IL1-β (330 bp), MIP1 (368 bp), MIP2 (325 bp), TNF-α (795 bp), iNOS (314 bp), IL-6 (474 bp), COX-2 (470 bp), GM-CSF (508 bp), KC (391 bp), IP10 (127 bp), and MCP-1 (271 bp). The following oligonucleotides were used as primers. The IL-1β primers were 5′-CTG TCC TGA TGA GAGCAT CC-3′ (sense) and 5′-TGT CCA TTG AGG TGG AGA GC-3′ (antisense). The MIP-1 primers were 5′-ATG AAG CTC TGC GTG TCT GC-3′ (sense) and 5′-TGA GGA GCA AGG ACG CTT CT-3′ (antisense). The MIP-2 primers were 5′-ACA CTT CAG CCT AGC GCC AT-3′ (sense) and 5′-CAG GTC AGT TAG CCT TGC CT-3′ (antisense). The TNF-α primers were 5′-GTT CTG TCC CTT TCA CTC ACT G-3′ (sense) and 5′-GGT AGA GAA TGG ATG AAC ACC-3′ (antisense). The iNOS primers were 5′-CTG CAC TTG GAT CAG GAA CCT G-3′ (sense) and 5′-GGG AGT AGC CTG TGT GCA CTT GAA A-3′ (antisense). The IL-6 primers were 5′-CAT GTT CTT CTG CAA TTC ACT GCT CTA ACC-3′ (sense) and 5′-GGT AGA GAA TGG ATG AAC ACC-3′ (antisense). The GM-CSF primers were 5′-TCT GGT CTC GCA TCA ATG GGA ACC CTT G-3′ (antisense) and 5′-TAT CTC GGT CGT GCT GTA AAG TCT GAT CTA GAG GAA CCG CTT GGT GGA TTG GAA TGA ACC ACC-3′ (antisense).

FIGURE 5. Effects of SET-M33 peptide on wound closure in a monolayer of HaCaT cells. A, wounds at 24 h photographed by phase contrast light microscopy in control wells (left panel) and in wells incubated with SET-M33 1 μM (right panel). B, histograms of the cell-covered area (percentage) in wells without SET-M33 (left column) and with SET-M33 (right column) obtained using Tscratch software. All data are the mean ± S.D. of three independent experiments. ***, p < 0.0001.
An LPS-neutralizing Drug Candidate

Animal procedures were approved by the Italian Ministry of Health on September 21, 2012. BALB/c mice (Charles River), 8 weeks old and weighing ~20 g, were used for the experiment. The animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and internal guidelines. Prior to experimentation, all mice were allowed at least 4 days of acclimation following shipment.

Mice were anesthetized with Zoletil (0.2 ml/kg) plus Xilor (0.2 ml/kg) and treated with 25 μg/kg LPS from P. aeruginosa (serotype 10, strain ATCC 27316, Sigma-Aldrich, L 9143) and 5 mg/kg SET-M33, dissolved in saline solution, through an intratracheal aerosol sprayer (MicroSprayer® 1A-1C, Penn Century). Eight hours after intratracheal nebulization, mice were anesthetized and sacrificed by CO₂ inhalation for collection of bronchoalveolar lavage (BAL). BAL was collected by introduction of 1 ml of saline solution into the lungs via a 22-gauge catheter connected to a syringe. TNF-α concentrations in BAL were assessed by ELISA (ELISA MAX™ Deluxe Sets, BioLegend, San Diego, CA) according to the instructions of the manufacturer.

The data were processed by one-way analysis of variance and Dunnett post tests using GraphPad Prism 5.0 software. The levels of statistical significance are indicated in the figure legends.
Migration Assay

The peptide ability to stimulate migration of epithelial cells in vitro was studied according to a modified scratch assay (25–27). Briefly, HaCaT cells (2 × 10^5 cells/well) were seeded on each side of a culture insert for live cell analysis (Ibidi, Munich, Germany). Inserts were placed in wells of a 24-well plate and incubated at 37 °C under 5% CO₂ to allow cells to grow to confluence. Afterward, the inserts were removed with sterile tweezers to create a cell-free area (wound area) of ~500 μm. The cells were treated with 1 μM SET-M33 peptide in DMEM supplemented with 5% FBS and allowed to migrate in a suitable incubator. At time 0 and after 24 h (T0 and T24), wound areas were visualized under an inverted microscope (Zeiss microscope) at ×4 magnification and photographed with a Nikon ACT-1 version 2.63 camera. The percentage of cell-covered area at the two times was determined by Tscratch software. The p value was calculated by one-tailed Student’s test using GraphPad Prism 5.0 software, and the value is reported in the figure legends.

Author Contributions—J. B. and A. P. coordinated the whole project and wrote the article. C. F. and L. B. synthesized and purified peptides. J. B., G. R., and L. Q. performed in vitro experiments for migration and gene expression of inflammatory cytokines and in vivo LPS neutralization. I. L. and R. G. performed the Bio-plex analysis.

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