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RESEARCH ARTICLE

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An antimicrobial molecule mitigates signs of sepsis in vivo and eradicates infections from lung tissue

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Abstract

The peptide sequence KKIRVRLSA was synthesized in a dimeric structure (SET-M33DIM) and evaluated as a candidate drug for infections due to multidrug-resistant (MDR) Gram-negative pathogens. SET-M33DIM showed significant antibacterial activity against MDR strains of *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Escherichia coli* (Minimal Inhibitory Concentration [MICs], 1.5-11 μM), and less activity against *Pseudomonas aeruginosa* (MICs, 11-22 μM). It showed very low toxicity in vitro, ex vivo, and in vivo; in cytotoxicity tests, its EC_{50} was as much as 22 times better than that of SET-M33, a peptide with the same amino-acid sequence, but synthesized in tetra-branched form (638 vs 28 μM). In in vivo and ex vivo experiments, SET-M33DIM cleared *P. aeruginosa* infection, significantly reducing signs of sepsis in animals, and restoring cell viability in lung tissue after bacterial challenge. It also quelled inflammation triggered by LPS and live bacterial cells, inhibiting expression of inflammatory mediators in lung tissue, cultured macrophages, and bronchial cells from a cystic fibrosis patient.

KEYWORDS

antibacterial agents, antibiotic resistance, antimicrobial peptides, drug development

Abbreviations: AMP, antimicrobial peptides; Boc, tert-butoxycarbonyl; BSA, albumin bovine serum; CalceinAM, calcein acetoxymethyl; CFU, colony-forming units; DIPEA, N,N-disopropylethylamine; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, N,N,N,N-tetramethyluronium hexafluorophosphate; HDPs, host defense peptides; i.p., intraperitoneal; i.t., intratracheally; i.v., intravenous; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MDR, multidrug resistant; MEM, minimum essential medium w/Earle's salt; MH, Mueller-Hinton; MIC, minimal inhibitory concentration; PB, phosphate buffer; PCLS, precision-cut lung slice; SEM, scanning electron microscopy; tBu, tert-butyl ether; TCA, trichloroacetic acid; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TFacetate, trifluoroacetate.

1 | INTRODUCTION

Antimicrobial resistance is a major threat to human health and deserves the attention of governments around the world.[1] Significant efforts to develop novel antibacterial agents have produced many new molecules. However, since few antimicrobial compounds have been approved for clinical use in the last 20 years, resistant bacteria have continued to spread.[2-4]

Possible new therapies in the pipeline include antimicrobial peptides (AMP) which are particularly promising for the treatment of bacterial infections,[5-10] since they combine antimicrobial activity with angiogenic, immune modulating, and anti-inflammatory properties.[11] Current antibiotics based on small molecules rarely have these features.[12]

Natural AMP, also known as host defense peptides (HDPs), are a diverse class of molecules that offer first-line defense against dangerous microbes and have significant activity against Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and parasites.[13-17] They also show a unique combination of immune-stimulant and anti-inflammatory properties.[18-22]

Few peptide molecules are currently used in clinical practice[7] due to their general toxicity, short half-life and in rare cases, production difficulties. Nevertheless, hundreds of AMPs are described in the literature as possible new drugs, several are in preclinical development and some are close to clinical trials.

SET-M33 is an antimicrobial peptide that has been extensively studied in recent years.[23-28] It is a non-natural cationic peptide built in tetra-branched form.[29] This structure makes the molecule more resistant to degradation in biological fluids.[30-32] It has shown efficacy against a number of Gram-negative multidrug-resistant clinical isolates.[23,26,28] Its mechanism of action is based on binding to LPS and disruption of bacterial membranes.[33,34] SET-M33 is in the late stage of preclinical characterization prior to clinical trials.

To identify back-up molecules, we are testing a panel of modified versions of SET-M33 with the intention to obtain new molecules with better performance in terms of pharmacological profile. Here we report a peptide with the same amino acid sequence (KKIRVRLSA) but synthesized in two-branched dimeric form (SET-M33DIM) instead of the tetra-branched structure of the original SET-M33.

2 | MATERIALS AND METHODS

2.1 | Peptide production

SET-M33DIM peptide was synthesized on a Syro automated machine (MultiSynTech, Witten, Germany) using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and activation of the carboxylic group with O-(benzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexafluorophosphate (HBTU)/N,N-disopropylethylamine (DIPEA). Side-chain-protecting groups were tert-butoxycarbonyl (Boc) for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, and tert-butyl ether (tBu) for Ser. SET-M33DIM was synthesized on Tentagel S RAM resin with Fmoc-βAla-OH as first coupling step. Fmoc-Lys(Fmoc)-OH was then used to build the dimeric core, followed by nine sequential additions of Fmoc amino acids to complete the peptide KKIRVRLSA. The final product was cleaved from the solid support, deprotected by treatment with TFA containing triisopropylsilane and water (95/2.5/2.5), and precipitated with diethyl ether. Crude peptide was then purified by reversed-phase chromatography on a preparative X-Bridge BEH Waters C18 column (300 Å, 10 μm, 250 × 20 mm) in linear gradient form for 40 min, using 0.1% TFA/water as eluent A and acetonitrile as eluent B. Due to the cleavage and purification conditions, the purified peptide was obtained as a trifluoroacetate (TFacetate) salt. The exchange from TFacetate (toxic) to acetate was carried out using a quaternary ammonium resin in acetate form (AG1-X8, 100-200 mesh, 1.6 meq/g capacity, Bio-Rad). We used a resin-to-peptide ratio of 1000:1 to ensure high anion exchange yield. The resin was added directly to the peptide solution and the suspension was stirred for 1 hour. The peptide sample was filtered and recovered. The resin was washed extensively with water and the pooled solutions were evaporated and freeze-dried.

Peptide purity and identity were confirmed by reversed-phase chromatography on a Phenomenex Jupiter C18 analytical column (300 Å, 5 μm, 250 × 4.6 mm) and by mass spectrometry with a Bruker Daltonics Ultraflex III MALDI TOF/TOF.

The tetra-branched peptide used here for comparison was synthesized as previously described.[26]

2.2 | Minimal inhibitory concentration assay

Minimal inhibitory concentrations (MICs) were determined in triplicate using a standard microdilution assay as recommended by the Clinical and Laboratory Standards Institute, with cation-supplemented Mueller-Hinton (MH) broth (Becton Dickinson, Franklin Lakes, NJ, USA) and a bacterial inoculum of 5×10^4 CFU/well in a final volume of 100 μL. The results were recorded by visual inspection after 18-20 hours of incubation at 37°C.

2.3 | In vivo efficacy

Animal procedures were approved by the Italian Ministry of Health, 14th January 2016, protocol 34/2016-PR. Eight-week-old BALB/c female mice (Charles River) were used in

all experiments. 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. For the sepsis model, the animals (20 g) were rendered neutropenic by intraperitoneal (i.p.) administration of cyclophosphamide (C7397 Sigma-Aldrich, St. Louis, USA) at 150 mg/kg (300 µL of 10 mg/mL solution) 4 days and 1 day before infection. Sepsis was induced by infecting animals i.p. with a lethal amount of *Pseudomonas aeruginosa* PAO1 (0.6×10^3 CFU/mouse) in 500 µL PBS. The mice were treated three times with i.p. injection of SET-M33DIM, diluted in 0.9% NaCl solution, at 5 mg/kg and 10 mg/kg, 10 minutes, 6 and 24 hours post-infection. Control animals received only vehicle. Moribund animals were killed humanely to avoid unnecessary distress. For the lung infection model, animals (20 g) were first anesthetized with Zoletil 50/50 (250 mg tielamine + 250 mg zolazepam) + nerfastin (20 mg xilazine). Then, they were infected with *P. aeruginosa* PAO1 1.5 x 10^6 dissolved in 25 µL of 0.9% NaCl solution, which was delivered through the trachea (i.t.) with a Hamilton syringe. After 25-30 minutes from the infection, the animals were treated i.t. with the peptide (5 mg/kg and 10 mg/kg) diluted in 0.9% NaCl solution, using a Penn Century device (FMJ-250, Penn-Century Inc, USA) for nebulization. 4 hours post-infection, animals were sacrificed with CO_2 and the lungs collected and homogenized in 2 mL of sterile solution (PBS) using a "Tissue Ruptor" homogenizer with sterile probes (Qiagen, Hilden, Germany). Samples were diluted serially and 100 µL of each dilution was spread in duplicate on appropriate agar plates for colony count.

2.4 | Stability

SET-M33DIM diluted to 1 mg/mL in DMSO was incubated at 37°C with a solution of RPMI-1640 medium supplemented with 25% (v/v) human serum. Five tubes were prepared containing 200 µL of the diluted serum and 50 µL of peptide solution. For the precipitation of serum proteins, 50 µL of cold (+4°C) 7.5% aqueous trichloroacetic acid (TCA) was added to each reaction solution at different time intervals (0, 2, 24, 48, and 72 hours), and then centrifuged at 13 000 *g* for 5 minutes.

270 µL supernatant was collected and diluted with 430 µL 1% trifluoroacetic acid (TFA)/water. Each sample was then analyzed by RP-HPLC and mass spectrometry to detect uncleaved peptide or fragments. RP-HPLC was performed on a Phenomenex Jupiter C18 analytical column (300 Å, 5 µm, 250 × 4.6 mm), using 0.1% TFA/water as eluent A and acetonitrile as eluent B in a linear gradient. MS analysis was performed with a Bruker Daltonic ultraflex MALDI TOF/TOF mass spectrometer. The experiments were performed in triplicate.

2.5 | Cytotoxicity

16HBE14o- (human bronchial epithelial cells) were plated at a density of 2.5×10^4 per well in 96-well microplates, previously incubated with coating solution (88% LHC basal medium, 10% bovine serum albumin, 30 µg/mL bovine collagen type I, and 1% human fibronectin). Different concentrations of SET-M33 or SET-M33DIM, from 0.05 to 50 mol/L, diluted in culture medium (Minimum Essential Medium w/ Earle's salt [MEM], 10% FBS, penicillin [100 U/mL], streptomycin [10 000 mcg/mL], glutamine [2 mM]), were added 24 hours after plating. Cells were grown for 48 hours at 37°C. Viability was assessed with 0.1% crystal violet solution. EC_{50} values were calculated by non-linear regression analysis using GraphPad Prism 5.03 software.

2.6 | Electron microscopy

16HBE14o- (human bronchial epithelial cells) were plated at a density of 3.0×10^4 per well in 24-well microplates, grown for 24 hours and then incubated with 20 µM SET-M33 or SET-M33DIM in appropriate culture medium at 37°C for 48 hours.

2.6.1 | Scanning electron microscopy

After washing in PBS, the cells were fixed in 2.5% glutaraldehyde solution in phosphate buffer 0.1 M pH 7.2 (PB) for 2 hours at 4°C, washed in PB, post-fixed in 1% OsO_4 in PB for 30 minutes at 4°C, dehydrated in an ascending alcohol series, and dried in a Balzers CPD 030 CO_2 critical point dryer. The coverslip was then mounted on an aluminum stub, coated with 20 nm gold in a Balzers MED010 sputtering device, and observed in a Philips XL20 scanning electron microscope at an electron accelerating voltage of 20 kV.

2.6.2 | Transmission electron microscopy

The cells were fixed in 2.5% glutaraldehyde solution in phosphate buffer 0.1 M pH 7.2 (PB) for 2 hours at 4°C, washed in PB, post-fixed in 1% OsO_4 in PB for 30 minutes at 4°C, dehydrated in an ascending alcohol series, incubated twice in propylene oxide and finally infiltrated and embedded in epon/araldite resin that was polymerized at 60°C for 48 hours. Ultrathin sections (60 nm thick) were cut from samples on a Reichert-Jung Ultracut E ultramicrotome. They were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and observed in a FEI Technai G2 SPIRIT transmission electron microscope at an electron accelerating voltage of 100 kV under standard operating conditions.

2.7 | Hemolytic assay

Whole human blood in EDTA was centrifuged (1100 g) for 10 minutes. Red blood cells diluted 1:100 in physiological solution (0.9% NaCl) were incubated for 24 hours at 37°C with two-fold serial dilution of SET-M33DIM from 3.9 mg/L to 8 g/L. The absorbance of the supernatants was determined in a 96-well plate at 490 nm with a microplate reader. Data for 100% hemolysis were obtained by adding 0.1% TritonX-100 in water to cells. The negative control was physiological solution. The hemolysis rates of the peptides were calculated with the following equation:

$$(\%) = \left(A_{\text{peptide}} - A_{\text{physiological solution}}\right) / \left(A_{\text{triton}} - A_{\text{physiological solution}}\right) \times 100\%$$

where A = absorbance.

2.8 | Acute toxicity in vivo

Animal care procedures were followed as described for efficacy in vivo. BALB/c female mice were treated by a single i.v. administration of different amounts (40, 20, and 10 mg/kg) of SET-M33 or SET-M33DIM, diluted in physiological solution (0.9% NaCl). Signs of toxicity were monitored four times a day by visual inspection. A toxicity score was assigned for the following signs: wiry coat and poor motility = mild signs; very wiry coat, abundant lachrymation, and poor motility even under stimulation = manifest signs. Animals were observed for 4 days after inoculation of the peptide. Mice were weighed every day from arrival to the last day of the experiment. Moribund animals were killed humanely to avoid unnecessary distress.

2.9 | LPS neutralization

RAW264.7 murine macrophages were seeded in six-well plates (5×10^5 cells per well) with complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 μg/mL glutamine, 100 μg/mL streptomycin, and 60 μg/mL penicillin) and cultured in a CO_2 incubator overnight. Cells were stimulated with 50 ng/mL lipopolysaccharide (LPS) from *Klebsiella pneumoniae* (serotype 10, strain ATCC 27316; SIGMA L 9143), or 20 ng/mL LPS from *K. pneumoniae* (strain ATCC 15380; SIGMA L 4268), or 20 ng/mL LPS from *Escherichia coli* (026:B6; SIGMA L 8274) in DMEM, in the presence of 50 μM, 20 μM, or 10 μM peptide SET-M33DIM in DMEM for 6 hours. RT-PCR was used to evaluate messenger RNA expression. Total RNA was extracted using a NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. 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), COX2 (470 bp), GM-CSF (508 bp), KC (391 bp), IP10 (127 bp), and MCP-1 (271 bp). The following oligonucleotides were used as primers: IL-1β primers were 5′-CTG TCC TGA TGA GAG CAT CC-3′ (sense), 5′-TGT CCA TTG AGG TGG AGA GC-3′ (antisense); MIP-1 primers were 5′-ATG AAG CTC TGC GTG TCT GC-3′ (sense), 5′-TGA GGA GCA AGG ACG CTT CT-3′ (antisense); MIP-2 primers were 5′-ACA CTT CAG CCT AGC GCC AT-3′ (sense), 5′-CAG GTC AGT TAG CCT TGC CT-3′ (antisense); TNF-α primers were 5′-GTT CTG TCC CTT TCA CTC ACT G-3′ (sense), 5′-GGT AGA GAA TGG ATG AAC ACC-3′ (antisense); iNOS primers were 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ (sense), 5′-GGG AGT AGC CTG TGT GCA CCT GGA A-3′ (antisense); IL-6 primers were 5′-CAT GTT CTC TGG GAA ATC GTG G-3′ (sense), 5′-AAC GCA CTA GGT TTG CCG AGTA-3′ (antisense); GM-CSF primers were 5′-TTT CCT GGG CAT TGT GGT CT-3′ (sense), 5′-AGT TCC TGG CTC ATT ACG CA-3′ (antisense); COX2 primers were 5′-TTT GCC CAG CAC TTC ACC CAT-3′ (sense), 5′-AAG TGG TAA CCG CTC AGG TGT-3′ (antisense); KC primers were 5′-ACT GCA CCC AAA CCG AAG TCA TAG-3′ (sense), 5′-GCA CAG TGG TTG ACA CTT AGT GGT-3′ (antisense); IP-10 primers were 5′-GCC GTC ATT TTC TGC CTC AT-3′ (sense), 5′-GCT TCC CTA TGG CCC TCA TT-3′ (antisense); MCP-1 primers were 5′-TTG GCT CAG CCA GAT GCA GTT A-3′ (sense), 5′-AAC TGC ATC TGC CCT AAG GTC TTC-3′ (antisense).

The following PCR conditions were applied: for IL1-β, MIP2, iNOS, and TNFα, 25 denaturing cycles at 94°C for 60 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 60 seconds; for MIP1 20 denaturing cycles at 94°C for 60 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 60 seconds; for KC and MCP1 30 denaturing cycles at 94°C for 60 seconds, annealing at 54°C (KC) and 55°C (MCP1) for 60 seconds and extension at 72°C for 60 seconds; for IP10 25 denaturing cycles at 94°C for 60 seconds, annealing at 54°C for 60 seconds and extension at 72°C for 60 seconds; for IL-6 and GM-CSF 30 denaturing cycles at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 60 seconds; for COX-2 25 denaturing cycles at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 60 seconds.

Densitometric analysis was carried out using ImageJ software

2.10 | Bio-Plex analysis

IB3-1 cells (LGC Promochem Europe), derived from a CF patient with a ΔF508/W1282X mutant genotype and immortalized with adeno12/SV40, were seeded in plates with LHC-8 medium supplemented with 5% FBS, in the absence of gentamicin, and cultured at 37°C/5% CO_2 overnight. Cells were initially pre-incubated with SET-M33 or SET-M33DIM

(2.5, 10, and 20 μM) for 24 hours. Then the medium was removed and *P. aeruginosa* non-mucoid laboratory strain PAO1 (Columbia University, New York, NY) (3×10^7 CFU/mL) and SET-M33 or SET-M33DIM (2.5, 10, and 20 μM) were added to fresh LHC-8 medium without FBS. Four hours after infection, the supernatants were collected and used for Bio-Plex cytokine, chemokine and growth factor assay (Bio-Rad Laboratories, Hercules, CA) as described by the manufacturer. The assay is designed for multiplexed quantitative measurement of multiple cytokines released in culture medium in a single well using as little as 50 μL of sample. Briefly, 50 μL of cytokine standards or samples (supernatants from treated cells spiked with 0.5% BSA) was incubated with 50 μL anti-cytokine-conjugated magnetic beads in 96-well plates for 30 minutes at room temperature with shaking. Plates were then washed three times with 100 μL Bio-Plex wash buffer using the Bio-Plex Pro Wash Station (Bio-Rad Laboratories); 25 μL of diluted detection antibody was added and the plates were incubated for 30 minutes at room temperature with shaking. After three washes, 50 μL of streptavidin-phycoerythrin was added and the plates were incubated for 10 minutes at room temperature with shaking. Finally, the plates were washed three times, the beads were suspended in Bio-Plex assay buffer, and the samples were analyzed on a Bio-Rad 96-well plate reader using the Bio-Plex Suspension Array System and Bio-Plex Manager software (Bio-Rad Laboratories).

2.11 | Precision-cut lung slice preparation

Female rats (Wistar, Crl:WI, Wu, nulliparous and non-pregnant) were obtained from Charles River (Sulzfeld, Germany). Animals were housed under conventional housing conditions (22 ± 2°C, relative humidity 55 ± 15%, 12 hours dark/light cycle). Diet and drinking water were available *ad libitum*. Animal health was checked daily. For preparation of Precision-cut lung slice (PCLS), animals were sacrificed by an i.p. overdose (~100 mg/kg body weight) of pentobarbital sodium (Narcoren, Merial GmbH, Hallbergmoos, Germany).

PCLS were prepared as previously described.[35,36] Briefly, the trachea was cannulated and the lungs were inflated with 37°C warm, 1.5% low-gelling agarose medium solution (Sigma-Aldrich, Munich, Germany) and cooled on ice. After polymerization of the agarose, 8 mm wide tissue cores were prepared and cut into 200- to 300-μm-thick slices using a Krumdieck microtome (Alabama Research and Development, Munford, AL, USA) and collected in 4°C cold EBSS (Sigma-Aldrich, Munich, Germany). The tissue slices were transferred to Petri dishes with DMEM/nutrient mixture F-12 Ham (pH 7.2-7.4) from Gibco (Life Technologies/Thermo Fisher Scientific, Dreieich, Germany) with l-glutamine and 15 mM HEPES, without phenol red or fetal bovine serum, supplemented with 100 units/mL penicillin and streptomycin (Lonza, Verviers, Belgium) and incubated under cell culture conditions (37°C, 5% CO_2). The medium was exchanged three times every 30 minutes to remove cell debris, and PCLS were incubated overnight (37°C, 5% CO_2).

2.12 | PCLS treatment for toxicity and antibacterial and anti-inflammatory activity

For efficacy and toxicity studies, one PCLS per well was placed in a 48-well plate and incubated overnight in DMEM. For cytotoxicity experiments, uninfected PCLSs were treated with SET-M33DIM at 24, 48, and 96 μM and in comparative wells, other PCLSs were treated with ciprofloxacin (Sigma, St. Louis, MO) at 15, 30, and 45 μM for 20 hours.

For antibacterial efficacy experiments, PCLSs were infected the next day with *P. aeruginosa* PAO1 (1×10^5 CFU/slice) by inoculation for 1h. Infected PCLSs were treated with SET-M33DIM at 24, 48, and 96 μM, up to 6 hours post-infection. After 6 hours, supernatants and lysed tissue, obtained by incubation with 250 μL 1% TX-100 in PBS, pH 7.4 plus 0.2% protease inhibitor cocktail for 1 hour, were collected for assessment of tissue viability, cytokines, and colony-forming units (CFU) determined by dilution plating on agar plates.

To assess anti-inflammatory activity of SET-M33DIM in infected PCLS, rat Interleukin-1beta (IL-1β) was measured using the Rat IL-1β DuoSet from R&D Systems (Wiesbaden-Nordenstadt, Germany) according to the manufacturer's specifications. For the eight-point standard curve, recombinant rat IL-1 β, included in the kit, was used. The lower and upper limits of quantification were at 16 pg/mL and 1000 pg/mL, respectively. OD was determined at 450 nm (reference wavelength 540 nm) using a Tecan reader.

2.13 | LDH assay

Lactate dehydrogenase (LDH) release was determined in PCLS supernatant using a Cytotoxicity Detection Kit PLUS (LDH) (Roche, Mannheim, Germany), according to the manufacturer's instructions. Briefly, 50 μL of tissue culture supernatant was incubated with 50 μL LDH reagent mix for 20 minutes at room temperature, protected from light. Absorbance was determined at a wavelength of 492 nm and reference wavelength of 630 nm using a Tecan reader. All tests were performed in duplicate. Triton X-100 lysed PCLSs were used as positive controls (100% LDH release).

2.14 | Calcein-AM staining

The viability of the tissue slices was assessed using Calcein acetoxymethyl (Calcein AM) staining (Life Technologies/Thermo Fisher Scientific, Dreieich, Germany) as previously described.[35,36] Briefly, PCLSs were incubated in 4 μM solution of Calcein-AM in DMEM-F12 for 45 minutes at 37°C and 150 rpm

in the dark. After washing three times with warm DMEM-F12 to remove excess dye, the slices were lysed with 1% TX-100 to release the dye from the cells. 50 µL of the lysate was transferred to a 96-well plate in duplicate and fluorescence was determined at wavelengths of 490 and 530 nm using a Tecan reader. The data were normalized to untreated PCLSs as a negative control (no loss of viability) set at 100%. PCLSs lysed with 70% EtOh were used as positive controls for cytotoxicity.

2.15 | Statistical analysis

All data were analyzed by GraphPad Prism 5.0 software using the following methods of calculation related to the different types of experiments: ANOVA with Dunnett post-test; Mantel-Cox test; non-linear regression; Student's *t* test. The relative p values are reported in the figure legends and pictures. The number of experiments used for statistical analyses is provided in figure legends.

3 | RESULTS

3.1 | Peptide synthesis

The two-branched peptide SET-M33DIM (Figure 1) was synthesized by solid phase synthesis. The identity and purity of the final product were confirmed by analytical reverse phase chromatography on a Jupiter C18 column and mass spectrometry. The product was more than 95% pure SET-M33DIM, as shown by HPLC (19.52 retention time) and mass spectrometry, with a peak at the expected molecular mass (2320 Da) (not shown).

3.2 | Therapeutic activity

3.2.1 | Minimal inhibitory concentrations

SET-M33DIM MICs were determined against strains of different bacterial species, including several Gram-negative pathogens (Table 1). Low MICs were observed against strains of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *E. coli*, and some other Enterobacterales, with the exception of *Proteus mirabilis*, *Serratia marcescens*, and *Burkholderia cepacia*. Satisfactory activity was found against *Pseudomonas aeruginosa*. Interestingly, SET-M33DIM activity was retained against MDR strains with various resistance mechanisms (such as extended spectrum β-lactamases and carbapenemases), including a MDR *P. aeruginosa* strain from a cystic fibrosis patient.

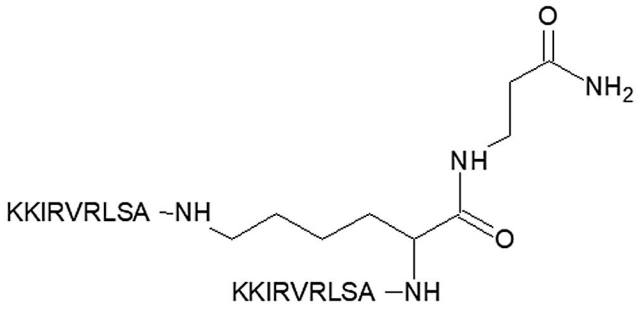


FIGURE 1 SET-M33DIM structure. Amino acids in the peptide sequences are reported as one-letter code. The two peptides are linked by a lysine core. The structure does not show actual proportions between peptide sequences and core

3.2.2 | Efficacy ex vivo

Rat precision-cut lung slices (PCLSs), obtained as described in Material and Methods, were infected with *P. aeruginosa* PAO1 (1×10^5 CFU/slice). One hour after infection, PCLSs were treated with SET-M33DIM 24, 48, and 96 µM up to 6 hours. At all concentrations, SET-M33DIM showed strong antimicrobial efficacy (Figure 2A). The reduction of bacterial load obtained with SET-M33DIM led to significantly improved lung tissue viability, as assessed by calcein viability staining. Infection with PAO1 without treatment resulted in massive loss of tissue viability, while treatment with SET-M33DIM resulted in significant improvement of tissue viability at all concentrations (Figure 2B).

3.2.3 | Efficacy in vivo

The peptide was tested in two animal models, an abdominal infection and a lung infection. For the abdominal infection, neutropenic mice (5/group) were injected i.p. with a lethal amount of *P. aeruginosa* PAO1 (0.6×10^3 CFU/mouse). They were then treated three times with SET-M33DIM i.p. at 5 mg/kg or 10 mg/kg at 0, 6, and 24 hours after infection and monitored for 4 days. While untreated mice had a mortality of 100% within 45 hours, we obtained 20% survival with SET-M33DIM 10 mg/Kg. When SET-M33DIM was used at 5 mg/kg, death was delayed by up to 45 hours with respect to the control group (Figure 3A). For the lung infection model, immunocompetent mice (6/group) were intratracheally (i.t.) infected with *P. aeruginosa* PAO1 (1.5×10^6 CFU/mouse) and, after 30 minutes treated i.t. with SET-M33DIM at 5 mg/kg or 10 mg/kg. Bacterial clearance was evaluated by counting CFUs recovered from lungs after 4 hours from infection. The median value of the two dosages showed an evident tendency to the dose dependency, with a strong statistical significance at 10 mg/kg (Figure 3B).

3.3 | Toxicity

3.3.1 | Cytotoxicity

SET-M33DIM was analyzed for toxicity to human bronchial epithelial cells (16HBE14o-). This cytotoxic assay showed

TABLE 1 MIC [μM] of SET-M33DIM against bacterial strains representative of several pathogenic MDR species

Species and strains	Features[a]	SET-M33DIM MIC [μM]
Pseudomonas aeruginosa ATCC 27853	Reference strain, wild type	11
P. aeruginosa PAO-1	Reference strain, wild type	11
P. aeruginosa VR-143/97	FQ^r AG^r ESC^r NEM^r (MBL/VIM-1)	>22
P. aeruginosa AV65	FQ^r AG^r ESC^r NEM^r (MBL/IMP-13)	>22
P. aeruginosa OBG6 1[a]	FQ^r AG^r ESC^r NEM^r (MBL/IMP-13)	22
P. aeruginosa CEFTO49	FQ^r AG^r ESC^r NEM^r (CARB/GES-5)	>22
Klebsiella pneumoniae ATCC 13833	Reference strain, wild type	5.5
K. pneumoniae 7086042	FQ^r AG^r ESC^r NEM^r (MBL/VIM-1)	3
K. Pneumoniae FI-47	FQ^r, AG^r, ESC^r, NEM^r, COL^r (CARB/KPC)	>22
K. Pneumoniae FI-20	FQ^r, AG^r, ESC^r, NEM^r, COL^r (CARB/KPC)	22
K. pneumoniae C8-27	FQ^r AG^r ESC^r ETP^r (ESBL/CTX-M-15)	11
K. pneumoniae FIPP-1	FQ^r AG^r ESC^r NEM^r (CARB/KPC-3)	22
Escherichia coli ATCC 25922	Reference strain, wild type	3
E. coli W03BG0025	FQ^r AG^r ESC^r (ESBL/CTX-M-15)	3
E. coli W03AN0048	FQ^r AG^r ESC^r (ESBL/CTX-M-15)	5.5
Proteus mirabilis W03VA1017	FQ^r ESC^r (AmpC/CMY-16)	>22
Enterobacter aerogenes W03BG0067	AG^r ESC^r (ESBL/SHV-5)	5.5
Enterobacter cloacae W03AN0041	ESC^r (ESBL/SHV-12)	5.5
Acinetobacter baumannii RUH 134	Reference strain, European clone II	3
A. baumannii RUH 875	Reference strain, European clone I	3
A. baumannii MR157	FQ^r AG^r ESC^r NEM^r (OXA/OXA-58)	3
A. baumannii N50-ColR	NEM^r COL^r (OXA/OXA-24)	22
Burkholderia cepacia ORB-99	Wild type	>22
Serratia marcescens W03BG0003	FQ^r AG^r ESC^r (ESBL/SHV-12)	>22

Note: Bacteria tested included reference strains (indicated) and clinical isolates (mostly with MDR phenotypes). The resistance traits and resistance mechanisms are indicated. AG^r, resistant to aminoglycosides (gentamicin, amikacin, and/or tobramycin); CARB, class A carbapenemase; COL^r, resistant to colistin; ESBL, extended spectrum-lactamase; ESC^r, resistant to expanded-spectrum cephalosporins; ETP^r resistant to ertapenem; FQ^r, resistant to fluoroquinolones; MBL, metallo-β-lactamase; NEM^r, resistant to carbapenems (imipenem and/or meropenem); OXA, oxacillinase. VIM-1, IMP13, and GES-5 are types of clinically important β-lactamases detected in the various strains.

[a]Clinical isolate with mucoid phenotype from patients with cystic fibrosis.

that SET-M33DIM has a considerably lower toxic effect on bronchial cells than SET-M33, the peptide in tetrameric form from which it is derived, as demonstrated by the higher EC_{50} of 638 μM compared to 28 μM for SET-M33 (Figure 4).

3.3.2 | Electron microscopy

The cytotoxic effects of SET-M33DIM and SET-M33 were compared using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Figure 5). Both peptides were incubated with 16HBE14o- bronchial epithelial cells at a concentration of 20 μM for 48 h. SEM observations did not detect any significant differences in cell shape or surface pattern between untreated cells (controls) and SET-M33DIM-treated cells (Figure 5A,B), while clear signs of cell damage were evident in cells incubated with SET-M33 (Figure 5C). Similarly, TEM observation of cell ultrathin sections did not detect any signs of cytoplasmic modifications compatible with cell damage in controls (Figure 5D,G) or SET-M33DIM-treated cells (Figure 5E,H): cell organelles showed canonical shape, location, and density. By contrast, SET-M33-treated cells showed partly disorganized cytoplasm richer in vesicles and with altered mitochondria and cell membrane damage (Figure 5F,I).

3.3.3 | Hemolytic activity

SET-M33DIM was also analyzed for its capacity to damage red blood cells (Figure 6). The peptide did not cause more than 22% hemolysis, even at a concentration of 8 mg/mL (2.7 mM), which is more than 100 times the highest MIC in Table 1.

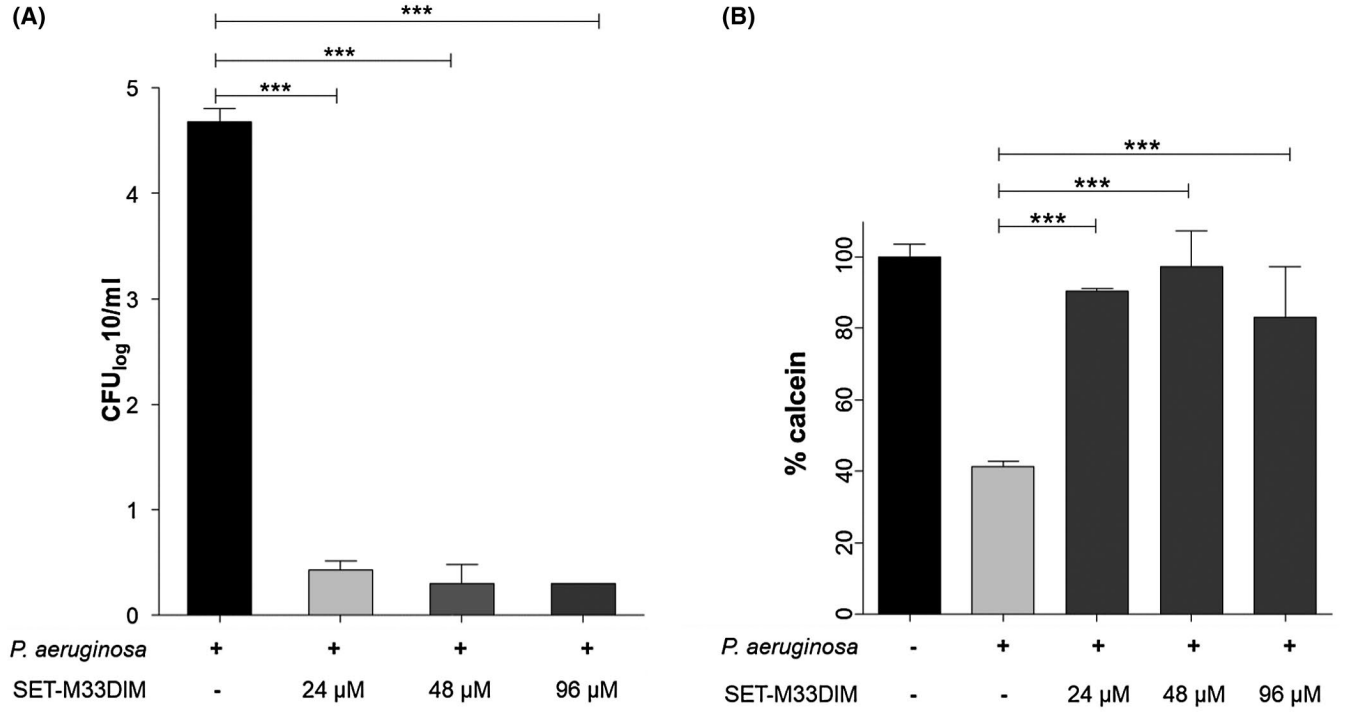


FIGURE 2 Ex vivo antimicrobial efficacy of SET-M33DIM on *P. aeruginosa*-infected rat PCLSs. A, Histograms indicate number of *P. aeruginosa* PAO-1 CFUs found in PCLSs. The black column shows CFUs in PCLSs 6 h after infection, without SET-M33DIM. The other columns show CFUs found in infected PCLSs 6 h after infection and treatment with SET-M33DIM at the concentrations indicated below. B, *Y* axis: percentage of calcein in PCLS cells. The black column shows the maximum cell viability in PCLSs not infected and not treated with the peptide (100%). The light grey column indicates maximum tissue damage in infected PCLSs without peptide treatment. The other columns indicate infected PCLSs treated with SET-M33DIM at different concentrations, as indicated below. Bars are mean + SD of n = 3. ***P value < .001 calculated by ANOVA with Dunnett post-test

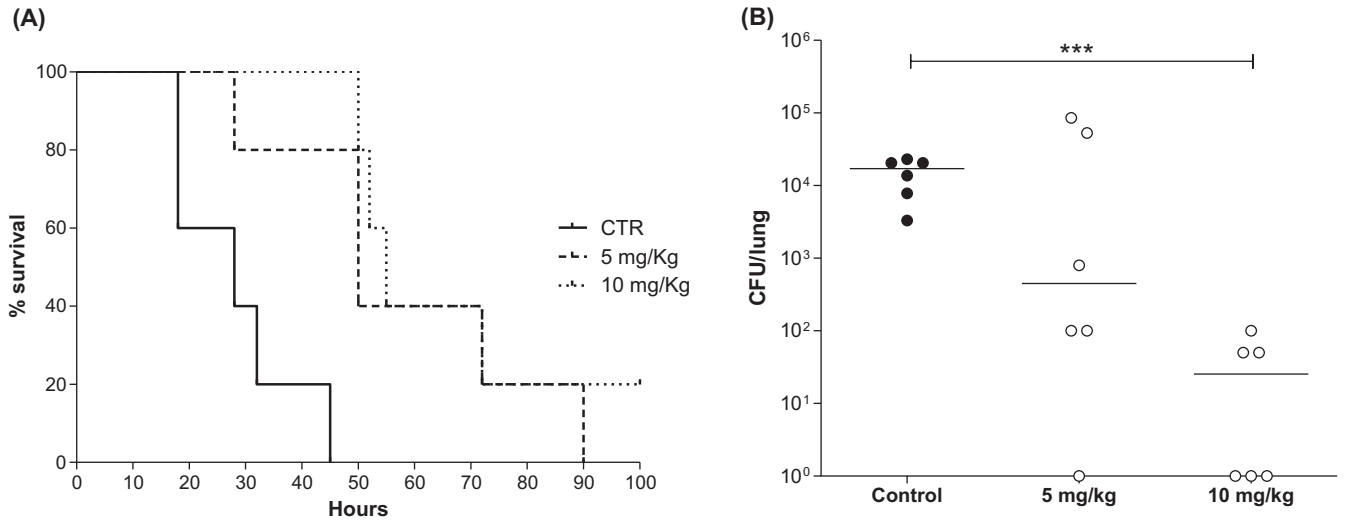


FIGURE 3 In vivo antibacterial activity of SET-M33DIM peptide. A, Sepsis model. BALB/c neutropenic mice (n = 5 per group) were injected i.p. with a lethal amount of *P. aeruginosa* PAO1 and then treated three times with SET-M33DIM (5 mg/kg or 10 mg/kg), 0, 6, and 24 h after infection. Survival percentage (*y*-axis) against time (*x*-axis) for the three groups; $P = .0055$ between survival curves calculated by the Mantel-Cox test. B, Lung infection model. Scatter plots representing the CFUs/lung (*y*-axis) in treated and untreated immunocompetent BALB/c mice (each circle corresponds to one mice, n = 6 per group). Animals were inoculated i.t. with *P. aeruginosa* PAO1 1.5×10^6, and after 30 min with SET-M33DIM 5 mg/kg or 10 mg/kg. Horizontal bars represent median values. ***$P < .001$ by Student's *t* test

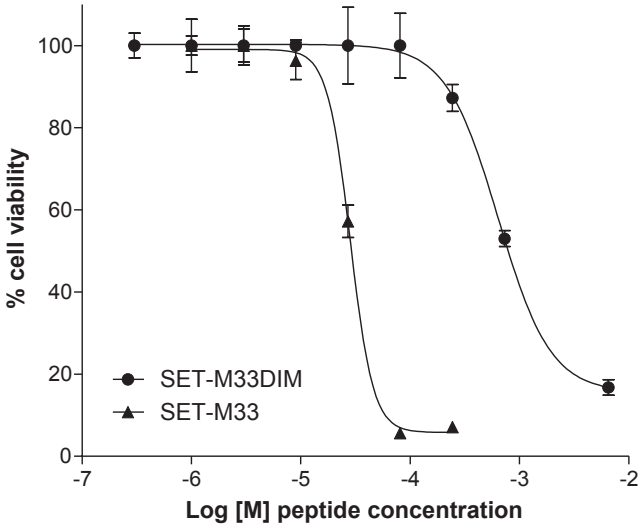


FIGURE 4 Cytotoxicity of SET-M33 and SET-M33DIM on 16HBE14o- bronchial cells. Cytotoxicity of SET-M33 (triangles) and SET-M33DIM (circles) in 16HBE14o- bronchial cells. Percentage cell survival (*Y*-axis) is plotted against peptide concentrations (*X*-axis) on a logarithmic scale. The data, reported as mean ± SD n = 3, was analyzed by non-linear regression

3.3.4 | Tissue viability

Uninfected viable lung slices were treated with ciprofloxacin 15, 30, and 45 μM or with SET-M33DIM 24, 48, and 96 μM for 20 hours. To evaluate tissue viability, LDH assay and Calcein AM staining were performed on PCLS supernatant and directly on PCLSs, respectively.

In the LDH assay, 1-hour treatment with Triton X-100, a common detergent, caused complete tissue death and was used as positive control (100% LDH release). The negative control was supernatant of untreated PCLS. After exposure to ciprofloxacin or SET-M33DIM, no significant effect on LDH release was observed with respect to the negative control (Figure 7A).

These results were confirmed by the Calcein AM staining test (Figure 7B). In this test, 15-minutes treatment with EtOH was used as toxicity control (not shown), since EtOH destroyed live tissue completely. Untreated PCLSs were used as negative control (100% viability). Again ciprofloxacin and SET-M33DIM 24 μM had no significant effect on tissue viability.

Only the highest concentration of SET-M33DIM caused a very weak loss of viability in both tests (LDH release 39.9% peptide vs 29.4% control; Calcein staining 91% peptide vs 100% control).

3.3.5 | Acute toxicity in vivo

SET-M33DIM was compared with the tetra-branched peptide SET-M33 for acute toxicity in vivo. BALB/c mice were injected i.v. with different amounts of SET-M33 or SET-M33DIM in the range 10–40 mg/kg, in a single dose (Figure 8), and were monitored for 4 days. Neither peptide proved lethal nor no signs of toxicity were observed up to 20 mg/kg (10 mg/kg groups are not shown in the figure). At 40 mg/kg, SET-M33 produced strong signs of toxicity and 90% mortality after 96 hours.[26] At the same dose, SET-M33DIM caused 60% mortality and clear signs of toxicity immediately after inoculation. These signs disappeared in live animals within 24 hours. No variation in body weight was detected (not shown).

3.3.6 | Stability in serum

The branched structure of peptides increases their stability to protease degradation.[30-32] However, stability depends on the state of ramification. The stability of SET-M33DIM and SET-M33 in serum was compared (Table 2). The dimeric peptide was still detected in serum up to 48 hours, although its degradation was more rapid than that of the tetrameric molecule, which was detected in higher percentages.

3.4 | Anti-inflammatory activity

3.4.1 | Gene expression of pro-inflammatory factors in macrophages

SET-M33DIM was tested for LPS neutralization in macrophages and consequently for its inhibitory effects on inflammatory cytokines. RAW264.7 murine macrophages were incubated with LPS from *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* or *E. coli*, and then treated with SET-M33DIM. The cytokines and enzymes analyzed were: TNF-α, IL-1β, IL-6, MIP-1, MIP-2, GM-CSF, KC, IP-10, MCP-1, COX2, and iNOS. Gene expression analysis by RT-PCR showed that stimulation of cells with LPS 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 then treated with SET-M33DIM (10, 20, or 50 μM as indicated in caption of Figure 9) the expression of pro-inflammatory cytokines and enzymes was inhibited as follows (Figure 9): 100% for MIP1 and COX-2 in cells stimulated with LPS from *P. aeruginosa* and for COX-2 in cells stimulated with LPS from *E. coli*; ≥85% for iNOS, GM-CSF and IP10 in cells stimulated with LPS from *P. aeruginosa*, for MIP1 and COX-2 in cells incubated with LPS from *K. pneumoniae*, for TNF-α and GM-CSF in cells incubated with LPS from *E. coli*; >60% for TNF-α, IL-1β, MIP2, and IL-6 in cells stimulated with LPS from *P. aeruginosa*, for TNF-α in cells incubated with LPS from *K. pneumoniae* and 30% for

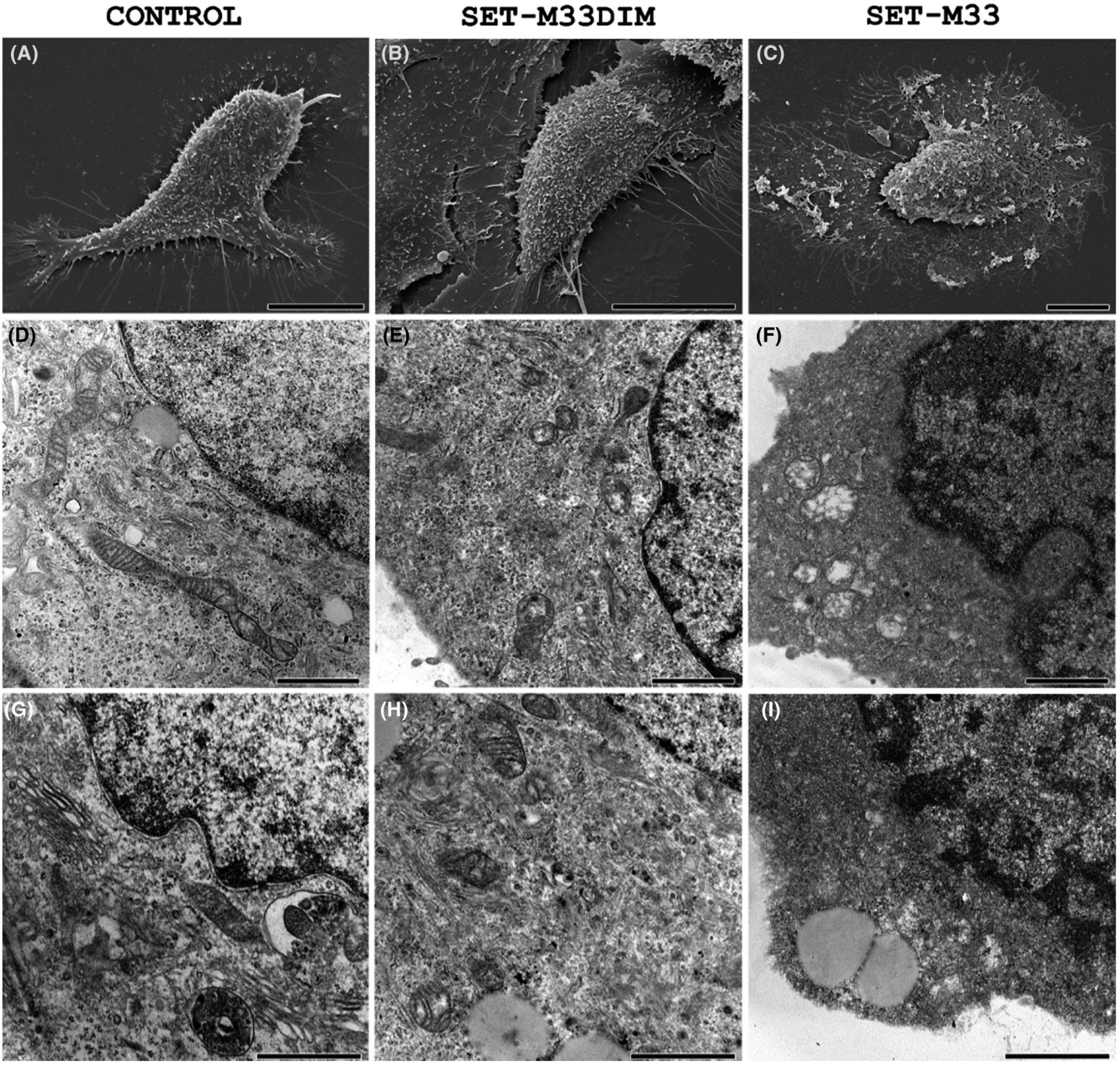


FIGURE 5 Scanning electron micrographs (SEM) and transmission electron micrographs (TEM). SEM (panels A-C) and TEM (panels D-I) of 16HBE14o- human bronchial epithelial cells. SEM and TEM micrographs of untreated cells (controls) (A, D, G) and cells after 48 h of incubation with SET-M33DIM (B, E, H) or SET-M33 (C, F, I). Scale bar 10 μm in panels A-C, and 500 nm in panels D-I

the other cytokines, except MCP1 stimulated by LPS from *P. aeruginosa* and *K. pneumoniae*, and IL-1β, KC, IP10, and MCP1 stimulated by LPS from *E. coli*, which were not inhibited at all.

3.4.2 | Inhibition of pro-inflammatory cytokines in *P. aeruginosa*-infected cystic fibrosis cells

The antibacterial effect observed in the ex vivo lung tissue slice infection model suggests that SET-M33DIM could potentially be used to treat chronic *P. aeruginosa* lung infections in cystic fibrosis patients. We analyzed inhibition of pro-inflammatory cytokines by SET-M33DIM and the original tetra-branched peptide SET-M33 in IB3-1 bronchial cells isolated from a cystic fibrosis patient.

The effects of SET-M33 and SET-M33DIM on the secretome profile of *P. aeruginosa*-infected IB3-1 cells were evaluated by Luminex technology,[37] detecting and quantifying a panel of proteins secreted into the medium. In this experiment, the pro-inflammatory stimulus was provided by whole live bacterial cells, unlike in the experiment described above, where soluble LPS was used. After pre-incubating

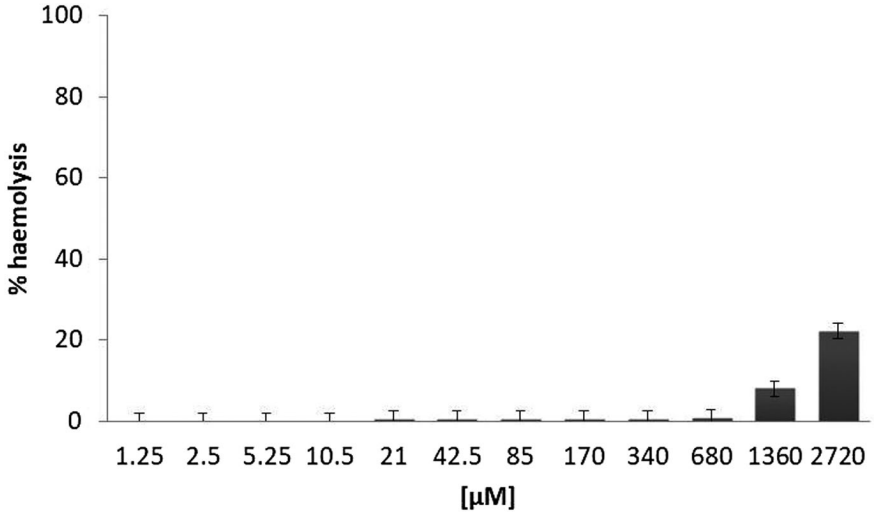
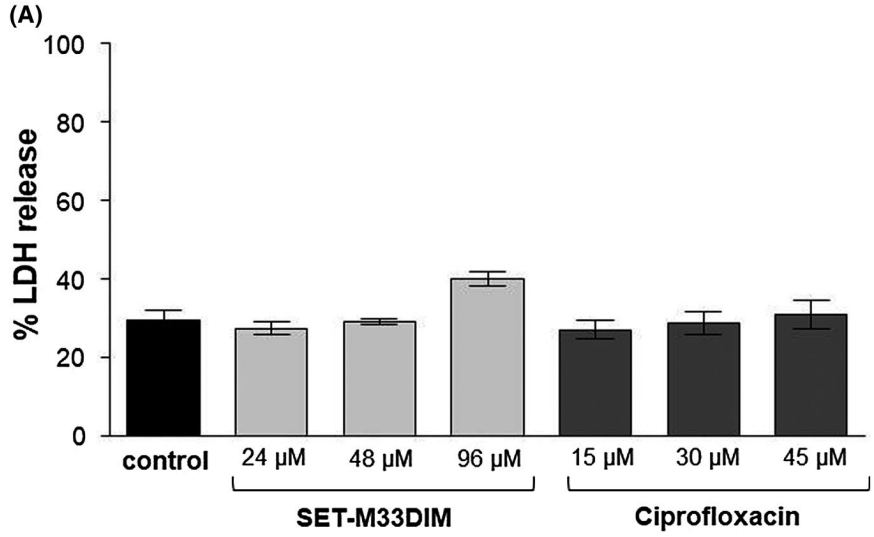


FIGURE 6 Hemolytic activity of SET-M33DIM. Hemolysis percentage (*y*-axis), calculated as described in Methods, against concentrations of SET-M33DIM (*x*-axis) of red blood cells incubated at 37°C for 24 h. Bars show mean ± SD for n = 2



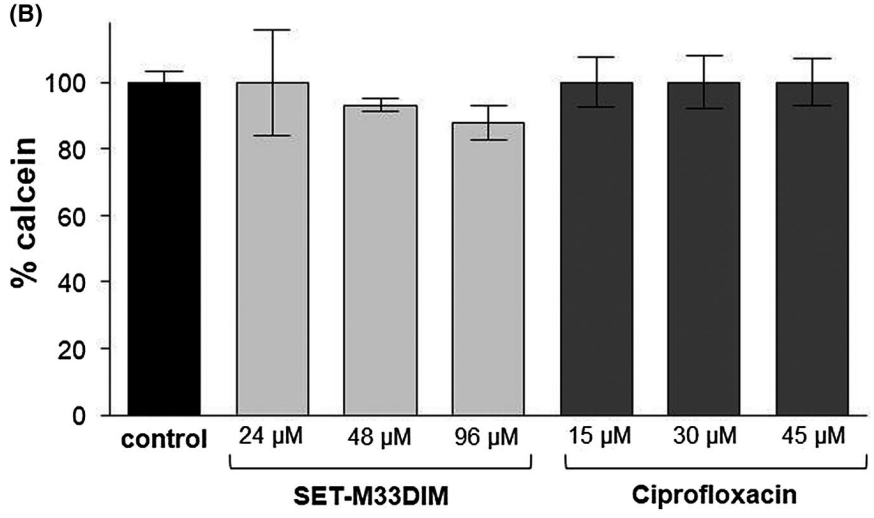


FIGURE 7 LDH assay (A) and Calcein AM staining (B) to measure PCLS viability after treatment with antibiotics. A, Tissue viability in rat PCLSs indicated by LDH release. 100% LDH release (maximum toxicity) was obtained after incubation with 1 h Triton X-100. PCLSs not treated with antibiotics (control black column) showed maximum viability. B, Cell viability of PCLSs indicated by Calcein staining. Untreated PCLSs (control black column) showed no toxicity (100% Calcein detection). PCLSs treated with EtOH (not shown) showed maximum toxicity (0%). Bars represent mean ± SD for n = 3 experiments

IB3-1 cells with the two peptides for 24 hours, the cells were infected for 4 hours with *P. aeruginosa* PAO1 strain and treated with SET-M33 or SET-M33DIM at different concentrations (Figure 10). Cells treated with 10 μM SET-M33DIM showed significant inhibition of G-CSF, IL-6, and VEGF (Figure 10A,B,D) with respect to cells incubated only

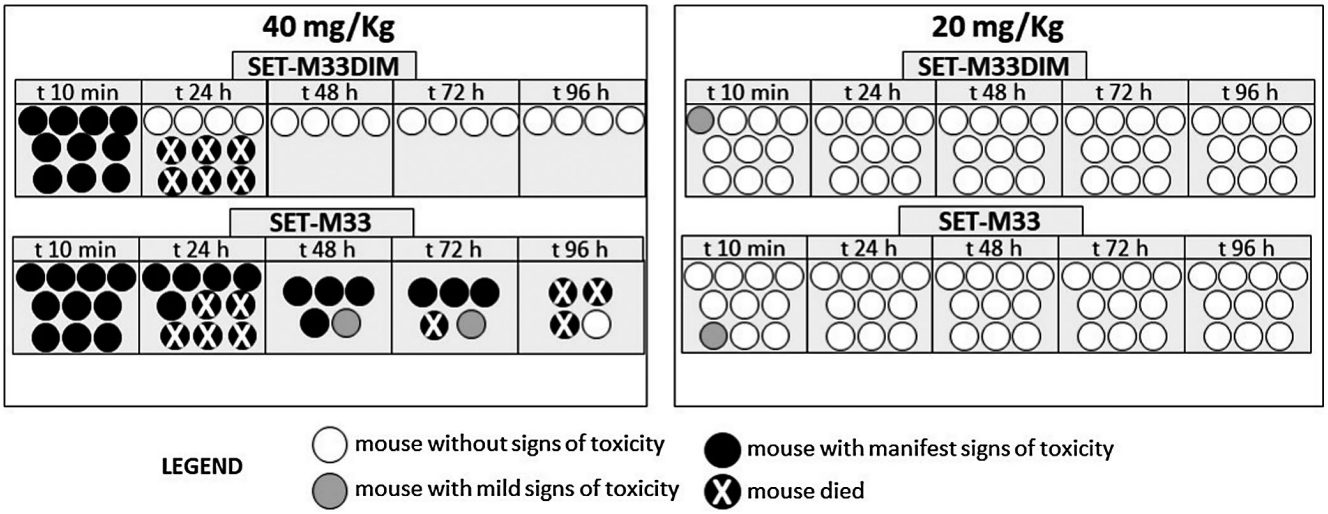


FIGURE 8 Acute toxicity of SET-M33 and SET-M33DIM in vivo. Mice (represented as circles) were inoculated i.v. with SET-M33 and SET-M33DIM at 40 and 20 mg/kg in a single dose, and monitored for 96 h. Different scales of grey and the X symbol in the circles indicate severity of signs or death, as described in the figure. The toxicity score is described in Methods

TABLE 2 Peptide stability measured as percentage of molecules detected in serum after incubation for 0, 2, 24, and 48 h. Data report the mean ± SD for n = 3 experiments

Peptide	0 h	2 h	24 h	48 h
SET-M33DIM	100% ± 0.00	79.13% ± 7.93	23.64% ± 9.53	7.20% ± 6.77
SET-M33	100% ± 0.00	89.65% ± 9.10	52.05% ± 6.68	49.33% ± 28.39

with *P. aeruginosa* PAO1. IL-8 was sensitively inhibited only when SET-M33DIM was used at 20 μM (Figure 10C). No significant differences between the two peptide activities were observed, except for the effect on IL-6, which proved to be strongly inhibited by all concentrations of SET-M33DIM, whereas SET-M33 only reduced protein secretion at the highest concentration.

3.4.3 | IL-1β as biomarker of inflammation in lung tissue

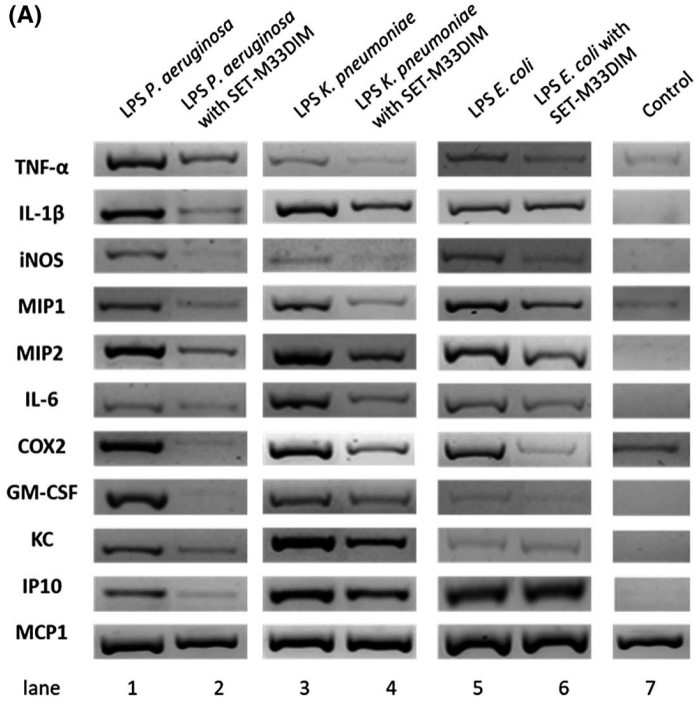
A further anti-inflammatory activity of SET-M33DIM was determined by measuring IL-1β expression ex vivo in lung tissue. Rat PCLSs were infected with *P. aeruginosa* PAO1 (1×10^5 CFU/slice) for 1 hour and treated with SET-M33DIM (24, 48, and 96 μM), 1 and 6 hours after infection. After 24 hours, culture supernatant was removed and the tissue lysed. IL-1β expressed in PCLS lysate was measured by ELISA. After treatment with SET-M33DIM, infected PCLSs showed the following inhibitions with respect to PCLSs incubated only with bacteria: 34% (24 μM), >54% (48 μM), and >86% (96 μM). SET-M33DIM alone did not trigger any IL-1β production (Figure 11).

4 | DISCUSSION

The pipeline of drug development is a long and complex process that includes lead identification, molecule optimization, preclinical characterizations in animals, setup of analytical methods, production scale-up, clinical trials, regulatory issues, and other procedures that must be conducted and passed prior to final approval for commercialization. Many, probably most, of the molecules in the industrial development pipeline fail a step in the preclinical or clinical stages, and are discontinued in their process toward the market. To increase the chances of approval of a new drug, companies therefore often have more than one molecule in their development pipeline for the same therapeutic indication.

The peptide SET-M33, a synthetic molecule produced in tetra-branched form, is currently in the late preclinical stage of development as a new drug to treat infections due to Gram-negative pathogens. Some back-up molecules are in earlier stages of development for the same indication, and the peptide SET-M33DIM is one of these.

Here we reported the in vitro, ex vivo, and in vivo characterization of SET-M33DIM, describing its toxicity in cells, tissues, and animals, and its antimicrobial and



(B)

Gene expression percentage			
	LPS *P. aeruginosa* with SET-M33DIM	LPS *K. pneumoniae* with SET-M33DIM	LPS *E. coli* with SET-M33DIM
TNF-α	25	21	8
IL-1β	30	69	91
iNOS	13	57	55
MIP1	0	15	37
MIP2	27	56	57
IL-6	21	46	52
COX-2	0	4	0
GM-CSF	15	73	15
KC	58	53	100
IP10	10	66	100
MCP1	100	100	91

FIGURE 9 Gene expression of pro-inflammatory cytokines and enzymes analyzed by RT-PCR. A, cDNA bands indicating gene expression in cells stimulated with LPS from *P. aeruginosa*, *K. pneumonia,* or *E. coli* (lanes 1, 3, 5, respectively), or in cells stimulated with LPS in the presence of SET-M33DIM (lanes 2, 4, 6, respectively). Control (lane 7) indicates a representative basal level of gene expression in unstimulated cells. B, Percentage of gene expression after LPS stimulation and SET-M33 treatment. The value 100% indicates gene expression in LPS-stimulated cells after subtraction of basal level (the real basal level obtained by each experiment, and not a representative one). Densitometric analysis was carried out using ImageJ software. RAW264.7 cells were incubated with SET-M33DIM 50 μM when stimulated with LPS from *E. coli* to measure inhibition of MIP2 and IL-6; when stimulated with LPS from *K. pneumoniae* to measure the inhibition of IL-1β, MIP2, iNOS, and MCP1; when stimulated with LPS from *P. aeruginosa* to measure the inhibition of IL-6, GM-CSF, MCP1, IL-1β, and iNOS. RAW264.7 were incubated with SET-M33DIM 10 μM when stimulated with LPS from *E. coli* to measure the inhibition of TNF-α and COX2. In all other cases, SET-M33DIM was used at 20 μM

anti-inflammatory activity, with a special focus on sepsis and lung infections. In some cases, comparative evaluations with the peptide in tetra-branched form (SET-M33) were necessary for a better understanding of efficacy and toxicity.

The dimeric form of the peptide showed slightly less antibacterial activity than the tetra-branched form (for a comparison of MICs, see[25]), though most bacterial species and strains showed similar sensitivity to the two peptides. However, the general toxicity of the dimeric form was significantly less, proving to be more than 22 times lower than that of the tetra-branched peptide in cytotoxicity tests. Experiments in vivo also demonstrated a sharp improvement in toxicity with less morbidity and mortality for SET-M33DIM with respect to the original peptide. Ex vivo study of live lung tissue (rat PCLSs) showed that SET-M33DIM had a toxicity profile comparable to that of the antibiotic ciprofloxacin, which is extensively used in clinical practice.

The clinical impact of ESKAPE pathogens (which include many Gram-negative bacteria)[38] and the urgent need for new drugs against these microorganisms in sepsis and lung infections prompted our evaluation of the capacity of SET-M33DIM to clear bacteria from sepsis in animals and infected lung tissue. In a sepsis animal model, the peptide caused an evident delay in the onset of signs of sepsis and death at doses compatible with clinical use, and in a lung infection model it produced a marked bacterial clearance in lungs of animals treated by nebulization. In the case of lung infections, we also used the technique of precision-cut lung slices, a new tool for preclinical respiratory research, which provides a link between cell-based in vitro experiments and complex in vivo animal models,[39] besides reflecting natural respiratory tract microanatomy. In rat lung tissue slices, SET-M33DIM strongly abated *P. aeruginosa* burden in a few hours, restoring lung cell viability practically to levels of non-infected tissue. In addition to antibacterial activity, SET-M33DIM showed strong anti-inflammatory activity, inhibiting expression of 14 cytokines, growth factors, and enzymes involved in inflammation, which we triggered in cells or tissues by incubation with soluble LPS from *E. coli*, *P. aeruginosa* or *K. pneumonia,* or by infection with living *P. aeruginosa* cells. Interestingly, the anti-inflammatory activity was observed in live lung tissue, macrophages, and human bronchial cells from a cystic fibrosis patient with

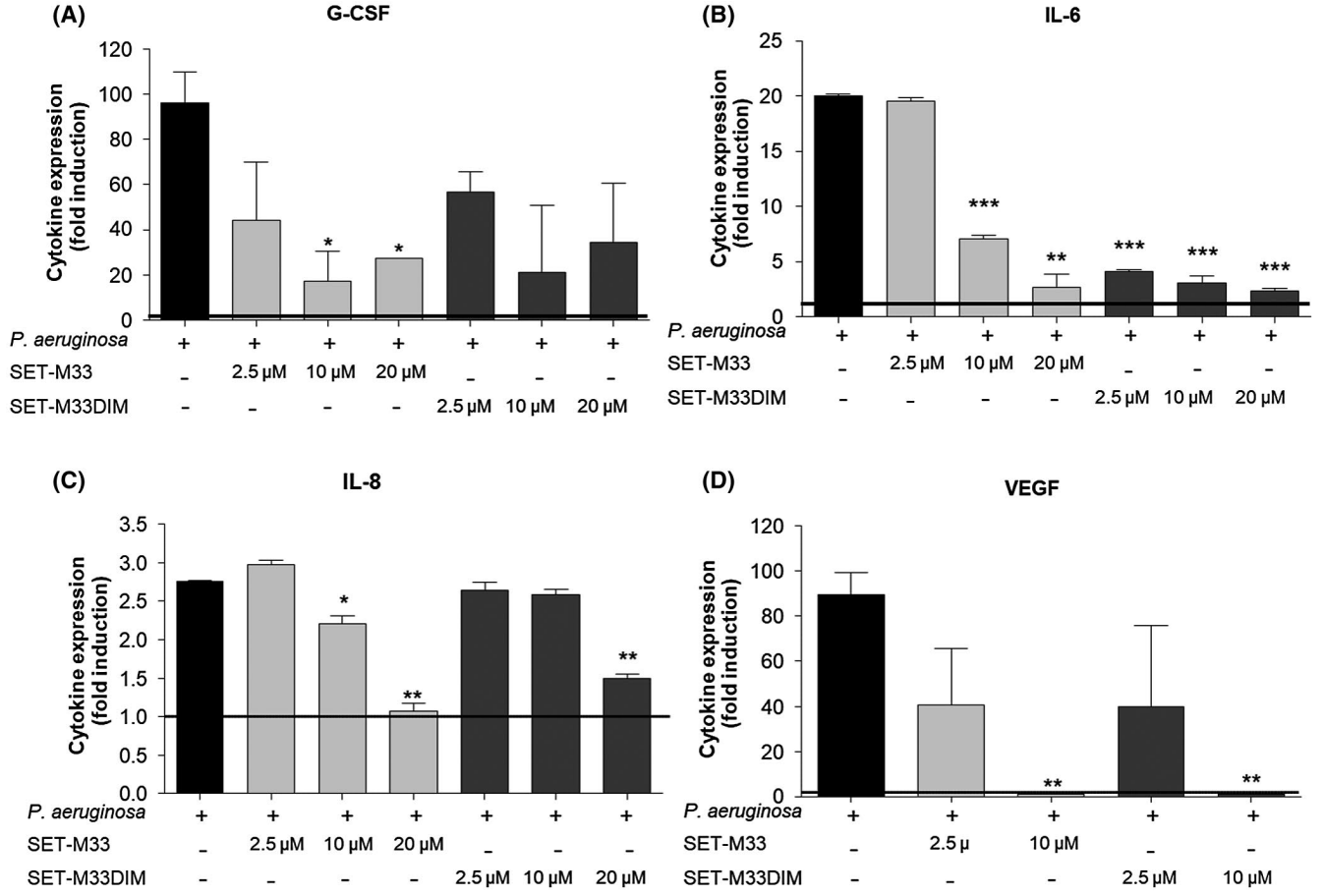


FIGURE 10 Analysis of expression of G-CSF (A), IL-6 (B), IL-8 (C), and VEGF (D) in IB3-1 cells incubated with *P. aeruginosa* and treated with SET-M33 or SET-M33DIM. Protein expression is reported in units of relative expression (fold induction) on the y-axis. Black columns indicate cells incubated with bacteria without peptide treatment (controls). Light grey columns are cells incubated with bacteria and treated with SET-M33 at the concentrations indicated. Dark grey columns are cells incubated with bacteria and treated with SET-M33DIM at the concentrations indicated. Basal levels of cytokine expression are indicated by horizontal black lines. Bars are mean ± SD of n = 3; ***$P < 0.001$, **$P < .01$, *$P < .05$ compared to controls by Student's *t* test

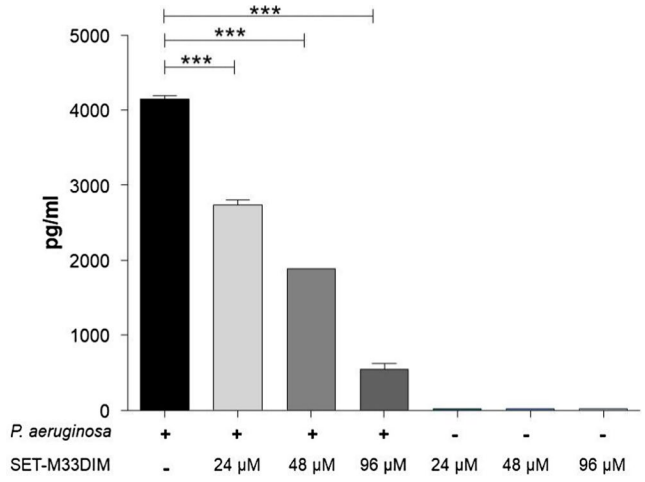


FIGURE 11 Effect of SET-M33DIM on IL-1β production in rat PCLSs infected with *P. aeruginosa* PAO1. Amount of IL-1β (pg/mL) in PCLS lysate after infection with PAO1 and treatment with SET-M33DIM 1 and 6 h later, analyzed 24 h post-infection. Bars are mean ± SDs for n = 3 experiments; ***$P < .001$ compared to infected PCLSs by ANOVA with Dunnett post-test

a heterozygous ΔF508/W1282X mutation genotype.[40] In cystic fibrosis, bacterial infections and inflammation of the lungs aggravate the general condition of patients, making the direct dual action (antibacterial and anti-inflammatory) of this peptide particularly welcome for possible treatment of the disease.

The characterizations of SET-M33DIM reported here make this molecule an important candidate lead compound for the development of a new drug to treat severe infections caused by MDR pathogens. Its low cost of synthesis, compared to other branched or dendrimeric peptides, makes the manufacturing process even more suited to industrial application for drug production.

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CONFLICT OF INTEREST

A.P., L.B., and C.F. are partners of Setlance, the company owing licences on the SET-M33 and SET-M33DIM patents.

AUTHOR CONTRIBUTIONS

A. Pini coordinated the whole project and wrote the article with the contribution of L. Bracci, C. Falciani, L. Quercini, and J. Brunetti; L. Quercini and S. Wronski performed ex-vivo cytotoxicity; J. Brunetti and G. Riolo performed in vitro cytotoxicity; S. Bindi performed the animal experiments; S. Scali synthesized and purified peptides; I. Lampronti and E. D'Aversa performed the bioplex analysis; M. Gentile and P. Lupetti performed the electron microscopy experiments; G.M. Rossolini and S. Pollini performed the MIC assay; J. Brunetti analyzed data and conducted statistical analyses.

REFERENCES

1. World Health Organization. *Antimicrobial Resistance: Global Report on Surveillance*. Geneva, Switzerland: World Health Organization; 2014.
2. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol*. 2010;8:251-259.
3. Andrei S, Valeanu L, Chirvasuta R, Stefan MG. New FDA approved antibacterial drugs: 2015–2017. *Discoveries*. 2018;6:e81.
4. Farha MA, Brown ED. Drug repurposing for antimicrobial discovery. *Nat Microbiol*. 2019;4:565-577.
5. Hale JD, Hancock RE. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev Anti Infect Ther*. 2007;5:951-959.
6. Jiang Z, Vasil AI, Vasil ML, Hodges RS. Specificity determinants improve therapeutic indices of two antimicrobial peptides piscidin 1 and dermaseptin S4 against the gram-negative pathogens *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Pharmaceuticals*. 2014;7:366-391.
7. Roscia G, Falciani C, Bracci L, Pini A. The development of antimicrobial peptides as new antibacterial drugs. *Curr Protein Pept Sci*. 2013;14:641-649.
8. Steckbeck JD, Deslouches B, Montelaro RC. Antimicrobial peptides: new drugs for bad bugs? *Expert Opin Biol Ther*. 2014;14:11-14.
9. Mahlapuu M, Hakansson J, Ringstad L, Bjorn C. Antimicrobial peptides: an emerging category of therapeutic agents. *Front Cell Infect Microbiol*. 2016;6:194.
10. Haney EF, Straus SK, Hancock REW. Reassessing the host defense peptide landscape. *Front Chem*. 2019;7:43.
11. Steinstraesser L, Kraneburg UM, Hirsch T, et al. Host defense peptides as effector molecules of the innate immune response: a sledgehammer for drug resistance? *Int J Mol Sci*. 2009;10:3951-3970.
12. Garrido-Mesa J, Rodríguez-Nogales A, Algieri F, et al. Immunomodulatory tetracyclines shape the intestinal inflammatory response inducing mucosal healing and resolution. *Br J Pharmacol*. 2018;175:4353-4370.
13. Boman HG. Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol*. 1995;13:61-92.
14. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol*. 1998;16:82-88.
15. Easton DM, Nijnik A, Mayer ML, Hancock RE. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol*. 2009;27:582-590.
16. Mansour SC, Pena OM, Hancock RE. Host defense peptides: front-line immunomodulators. *Trends Immunol*. 2014;35:443-450.
17. Brunetti J, Falciani C, Bracci L, Pini A. Models of in vivo bacterial infections for the development of antimicrobial peptide-based drugs. *Curr Top Med Chem*. 2017;17:613-619.
18. Marr AK, Gooderham WJ, Hancock RE. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr Opin Pharmacol*. 2006;6:468-472.
19. Hamill P, Brown K, Jenssen H, Hancock RE. Novel anti-infectives: is host defence the answer? *Curr Opin Biotechnol*. 2008;19:628-636.
20. Choi KY, Chow LN, Mookherjee N. Cationic host defence peptides: multifaceted role in immune modulation and inflammation. *J Innate Immun*. 2012;4:361-370.
21. Hancock REW, Haney EF, Gill EE. The immunology of host defence peptides: beyond antimicrobial activity. *Nat Rev Immunol*. 2016;16:321-334.
22. Silva ON, Porto WF, Ribeiro SM, Batista I, Franco OL. Host-defense peptides and their potential use as biomarkers in human diseases. *Drug Discov Today*. 2018;23:1666-1671.
23. Pini A, Falciani C, Mantengoli E, et al. A novel tetrabranched antimicrobial peptide that neutralizes bacterial lipopolysaccharide and prevents septic shock in vivo. *FASEB J*. 2010;24:1015-1022.
24. Pini A, Lozzi L, Bernini A, et al. Efficacy and toxicity of the antimicrobial peptide M33 produced with different counter-ions. *Amino Acids*. 2012;43:467-473.
25. Falciani C, Lozzi L, Scali S, Brunetti J, Bracci L, Pini A. Site-specific pegylation of an antimicrobial peptide increases resistance to *Pseudomonas aeruginosa* elastase. *Amino Acids*. 2014;46:1403-1407.
26. Brunetti J, Falciani C, Roscia G, et al. In vitro and in vivo efficacy, toxicity, bio-distribution and resistance selection of a novel antibacterial drug candidate. *Sci Rep*. 2016;6:26077.
27. Brunetti J, Roscia G, Lampronti I, et al. Immunomodulatory and anti-inflammatory activity in vitro and in vivo of a novel antimicrobial candidate. *J Biol Chem*. 2016;291:25742-25748.
28. Pollini S, Brunetti J, Sennati S, et al. Synergistic activity profile of an antimicrobial peptide against multidrug-resistant and extensively drug-resistant strains of Gram-negative bacterial pathogens. *J Pept Sci*. 2017;23:329-333.
29. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci USA*. 1988;85:5409-5413.
30. Bracci L, Falciani C, Lelli B, et al. Synthetic peptides in the form of dendrimers become resistant to protease activity. *J Biol Chem*. 2003;278:46590-46595.

31. Falciani C, Lozzi L, Pini A, et al. Molecular basis of branched peptides resistance to enzyme proteolysis. *Chem Biol Drug Des*. 2007;69:216-221.
32. Brunetti J, Falciani C, Bracci L, Pini A. Branched peptide as bioactive molecules for drug design. *Pept Sci*. 2018;2018:e24089.
33. Pini A, Giuliani A, Falciani C, et al. Characterization of the branched antimicrobial peptide M6 by analyzing its mechanism of action and in vivo toxicity. *J Pept Sci*. 2007;13:393-399.
34. Van der Weide H, Brunetti J, Pini A, et al. Investigations into the killing activity of an antimicrobial peptide active against extensively antibiotic-resistant *K. pneumoniae* and *P. aeruginosa*. *Biochim Biophys Acta Biomembr*. 2017;1859:1796-1804.
35. Neuhaus V, Danov O, Konzok S, et al. Assessment of the cytotoxic and immunomodulatory effects of substances in human precision-cut lung slices. *J Vis Exp*. 2018;9:135.
36. Niehof M, Hildebrandt T, Danov O, et al. RNA isolation from precision-cut lung slices (PCLS) from different species. *BMC Res Notes*. 2017;10:121.
37. Lampronti I, Borgatti M, Vertuani S, Manfredini S, Gambari R. Modulation of the expression of the pro-inflammatory IL-8 gene in cystic fibrosis cells by extracts deriving from olive mill waste water. *Evid Based Complement Alternat Med*. 2013;2013:960603.
38. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther*. 2013;11:297-308.
39. Neuhaus V, Schaudien D, Golovina T, et al. Assessment of long-term cultivated human precision-cut lung slices as an ex vivo system for evaluation of chronic cytotoxicity and functionality. *J Occup Med Toxicol*. 2017;12:13.
40. Tamanini A, Borgatti M, Finotti A, et al. Trimethylangelicin reduces IL-8 transcription and potentiates CFTR function. *Am J Physiol Lung Cell Mol Physiol*. 2011;300:L380-L390.

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