Safety evaluations of a synthetic antimicrobial peptide administered intravenously in rats and dogs

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The antimicrobial peptide SET-M33 is under study for the development of a new antibiotic against major Gram-negative pathogens. Here we report the toxicological evaluation of SET-M33 administered intravenously to rats and dogs. Dose range finding experiments determined the doses to use in toxicokinetic evaluation, clinical biochemistry analysis, necroscopy and in neurological and respiratory measurements. Clinical laboratory investigations in dogs and rats showed a dose-related increase in creatinine and urea levels, indicating that the kidneys are the target organ. This was also confirmed by necroscopy studies of animal tissues, where signs of degeneration and regeneration were found in kidney when SET-M33 was administered at the highest doses in the two animal species. Neurological toxicity measurements by the Irwin method and respiratory function evaluation in rats did not reveal any toxic effect even at the highest dose. Finally, repeated administration of SET-M33 by short infusion in dogs revealed a no-observed-adverse-effect-level of 0.5 mg/kg/day.

Rising antimicrobial resistance (AMR) is one of the greatest health challenges the world currently faces. It is estimated that approximately 700,000 deaths/year globally are due to drug-resistant bacteria1,2. It has been predicted that AMR could cause as many as 10 million deaths/year by 2050 with a global economic burden of USD 100 trillion3,4.

Although the need for new antibiotics is urgent, it seems that no single response is sufficient to fight AMR5,6. Considering the evolution of resistance to each new class of antibiotics introduced historically and the challenges involved in producing new antibiotics, focusing only on the research and development of new antibiotics is clearly insufficient5,8. A concerted global strategy is required.

Antimicrobial peptides (AMPs) are considered an interesting class of antibacterial molecule8–10. They cannot be considered a complete alternative to traditional antibiotics because they generally have lower activity and poor stability, and sometimes production difficulties11–13. However, they can play a very important role in the fight against bacteria because they are often active against bacteria resistant to traditional antibiotics14–16. Furthermore, some have a multifactorial mechanism of action: they kill bacteria and neutralize bacterial toxins, thus greatly reducing the inflammatory process triggered by living and dead bacteria17,18.

SET-M33 is a non-natural peptide synthesized in multiple antigen peptide form that makes it more stable in biological fluids19–21. SET-M33 has shown high antimicrobial activity in vitro and in vivo, anti-inflammatory activity, lack of immunogenicity and ability to eradicate biofilms22,23. Its mode of action features a two-step mechanism: (1) high affinity binding to LPS24 and (2) disruption of bacterial membranes25. Data on similar forms of the peptide, such as SET-M33D26, SET-M33DIM27, SET-M33Peg28 and SET-M33 encapsulated in dextran nanoparticles29, has been reported with in vivo activity as well. The peptide SET-M33 has completed preclinical development as a new antibacterial agent against major Gram-negative pathogens. Preclinical tests including CMC activities, ADME profile and in vivo efficacy in a murine model have been performed.

In this article we report the first toxicity results for SET-M33 administered intravenously in rats and dogs, two animal species recommended as rodent and non-rodent test systems, respectively, by international guidelines30,31. We report: dose range finding (DRF) in Sprague Dawley rats and beagle dogs; neurological toxicity and evaluation of respiratory function in rats; a 4-week toxicity study with 2-week recovery period in rats; 4-week toxicity study in dogs.
Table 1. Group mean values of significant changes obtained in haematology, clinical biochemistry and urinalysis tests (group mean values ± standard deviation) in male and female rats treated intravenously once daily for seven groups with 0 (vehicle = 0.9% saline solution), 10 and 20 mg/kg/day of SET-M33 peptide.

| Sex | Drug (mg/kg/day) | Retic (%) | Urea (mmol/L) | Creatinine (µmol/L) | Triglycerides (mmol/L) | Gluc (mmol/L) | Na (mmol/L) | Alb (g/dL) | Chol (mmol/L) | Ca (mmol/L) | Phos (mmol/L) | Total protein (g/L) | AlB (g/dL) | SG1 | Urinalysis |
|-----|-----------------|-----------|---------------|---------------------|-----------------------|--------------|-----------|----------|-------------|------------|-------------|----------------|-------------|-----|-----------|
| Male | 0               | 0.12 ± 0.03 | 0.14 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.03 |
|     | 10              | 0.06 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 | 0.08 ± 0.03 |
|     | 10              | 0.03 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 |
|     | 10              | 0.03 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.05 ± 0.03 |
|     | 10              | 0.02 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 |
|     | 10              | 0.02 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 | 0.04 ± 0.02 |

Results

Dose range finding (DRF) in rats. The purpose of this study was to determine the DRF of SET-M33 peptide when administered by intravenous bolus to Sprague Dawley rats. In order to identify a starting point for DRF, in Phase I of this study the peptide was administered once daily for 3 days at a constant dose of 20 mg/kg/day to six animals (three males and three females). Detailed observations were made daily during dosing: decreased or increased activity, circling, slow breathing, partially closed eyelids, swollen eyelids, abnormal uncoordinated gait, reduced body tone and hunched posture were observed in treated animals in the first 5 min. No clinical signs were recorded in any animal 20–60 min after administration. Body weight of all animals in Phase I remained constant or decreased slightly during treatment.

Phase II, where 10 animals/group (five males and five females) were used, included a control group and two dose groups (10 mg/kg/day and 20 mg/kg/day), that were injected once daily for seven days. Moderate clinical signs, such as partially closed eyelids, not perfectly coordinate gait and hunched posture, were observed at 10 mg/kg/day. At 20 mg/kg/day, additional clinical signs, including decreased activity, irregular and/or slow breathing, piloerection, partially closed eyelids, abnormal uncoordinated gait, reduced body tone and hunched posture were observed. Clinical signs were recorded in any animal 20–60 min after administration. Body weight of all animals in Phase II remained constant or decreased slightly during treatment.

Clinical laboratory investigations. Clinical haematology, biochemistry and urinalysis parameters were evaluated in animals in Phase II at the end of the treatment period. A complete list of parameters is reported in Materials and Methods. Administration of SET-M33 at 10 and 20 mg/kg/day for 7 days caused a statistically significant decrease of reticulocytes in males and females. SET-M33 caused a significant dose-related increase in creatinine levels in both sexes at 10 and 20 mg/kg/day and a significant increase in urea levels in both sexes at 20 mg/kg/day. There were significant differences in other parameters, such as glucose (males at 10 and 20 mg/kg/day), cholesterol (males at 20 mg/kg/day), triglycerides (females at 20 mg/kg/day), electrolytes, total protein and albumin (males at 20 mg/kg/day). Increased urine volume and lower specific gravity compared to the control group was recorded in both sexes at both doses. Differences were statistically significant for specific gravity in all cases and for volume in females at 20 mg/kg/day. Group mean values (± standard deviation) of significant changes are reported in Table 1.

Necroscopy. A gross necroscopy examination was performed on Phase I animals. A full necroscopy was performed on all Phase II animals. Organs were collected and weighed. No treatment-related findings were recorded at the end of Phase I. After Phase II, pale kidneys were observed in one female at 10 mg/kg/day and two females at 20 mg/kg/day. Dilated pelvis was observed in one male at 10 mg/kg/day. Higher kidney weight was recorded in both sexes at 20 mg/kg/day (26% and 36% increase with respect to the control group for males and females, respectively). A statistically significant dose-related decrease in heart weight was recorded in treated males compared to the control group. Prostate, seminal vesicles and coagulating gland weight from males dosed at 20 mg/kg/day was significantly lower than in the control group; no differences were recorded in the weight of the testes.

Bioanalytical and toxicokinetic study. SET-M33 concentration was determined after administration by intravenous bolus for 7 days at 10 mg/kg/day and 20 mg/kg/day. Exposure parameters (AUC and Cmax) were compared in order to evaluate dose-dependency, accumulation ratio and sex-related differences. On day 1, SET-M33 profiles showed quantifiable concentrations until 1 h post-dose and on day 7, until 0.5–1 h for 10 mg/kg/day and up to 24 h for 20 mg/kg/day. Mean plasma levels of SET-M33 increased in parallel with dose in males and females. Mean time to maximum concentrations (tmax) were observed immediately after administration, 5 min post-dose for both periods (days 1 and 7) and sexes, coherently with intravenous bolus administration. On day 1, mean
Female

| Sex  | Dose (mg/kg/day) | Hb (g/dL) | RBC (× 10^12/L) | MCV (fl) | MCH (pg) | MCHC (g/L) | MCV (fl) | MCH (pg) | MCHC (g/L) | PLT (× 10^9/L) | LUC | HDW (g/dL) | WBC (× 10^9/L) | SPT (sec) | SAPT (sec) |
|------|----------------|-----------|-----------------|---------|----------|-----------|---------|----------|-----------|---------------|-------|-----------|----------------|----------|-----------|
| Male | 0 0.458 ± 0.0136 | 15.2 ± 1.64 | 8.73 ± 0.399 | 0.122 ± 0.069 | 1.40 ± 0.153 | 17.4 ± 0.64 | 33.6 ± 0.74 | 51.6 ± 10.8 | 8.95 ± 0.818 | 3.06 ± 0.757 | 8.38 ± 1.371 | 0.06 ± 0.026 | 722 ± 58.2 | 21.5 ± 0.635 | 20.5 ± 0.346 |
| 5 0.262 ± 0.0187 | 8.9 ± 0.325 | 3.54 ± 0.167 | 0.004 ± 0.032 | 1.16 ± 0.197 | 17.1 ± 0.46 | 36.1 ± 0.98 | 40.6 ± 2.37 | 8.4 ± 0.95 | 3.02 ± 0.184 | 6.12 ± 0.43 | 0.05 ± 0.004 | 939 ± 130.1 | 21.3 ± 0.67 | 13.3 ± 0.41 |
| 9 0.431 ± 0.080 | 15.1 ± 0.29 | 8.84 ± 0.167 | 0.005 ± 0.015 | 1.03 ± 0.186 | 16.7 ± 0.29 | 34.9 ± 0.81 | 54.0 ± 1.12 | 7.67 ± 1.78 | 2.64 ± 0.102 | 6.12 ± 0.175 | 0.03 ± 0.014 | 81 ± 13.1 | 26.2 ± 0.133 | 20 ± 0.18 |
| 15 0.264 ± 0.086 | 8.9 ± 0.34 | 3.53 ± 0.129 | 0.005 ± 0.026 | 1.17 ± 0.405 | 17.3 ± 0.81 | 34.7 ± 0.115 | 7.98 ± 1.37 | 5.18 ± 0.72 | 5.98 ± 0.175 | 7.08 ± 1.15 | 0.05 ± 0.006 | 226 ± 108.6 | 21.9 ± 0.76 | 16.4 ± 1.15 |
| Female | 0 0.428 ± 0.0153 | 14.1 ± 0.47 | 7.62 ± 0.25 | 0.170 ± 0.045 | 2.31 ± 0.53 | 18.6 ± 0.46 | 33.7 ± 0.73 | 54.7 ± 1.64 | 7.30 ± 2.064 | 2.76 ± 0.102 | 6.25 ± 0.213 | 0.05 ± 0.023 | 853 ± 204.9 | 21.9 ± 1.18 | 37.0 ± 5.26 |
| 5 0.348 ± 0.0275 | 11.9 ± 0.84 | 6.81 ± 0.138 | 0.070 ± 0.026 | 1.10 ± 0.357 | 17.4 ± 0.50 | 32.6 ± 0.73 | 51.9 ± 1.71 | 11.36 ± 2.266 | 3.30 ± 1.734 | 10.30 ± 2.341 | 0.10 ± 0.046 | 937 ± 148.7 | 24.1 ± 0.84 | 31.9 ± 5.51 |
| 9 0.311 ± 0.0158 | 10.37 ± 0.56 | 6.15 ± 0.122 | 0.087 ± 0.017 | 0.49 ± 0.178 | 17.05 ± 0.56 | 36.0 ± 0.58 | 58.5 ± 1.77 | 13.51 ± 2.50 | 3.25 ± 1.551 | 12.35 ± 2.204 | 0.14 ± 0.054 | 918 ± 130.1 | 26.5 ± 1.89 | 31.9 ± 5.51 |

Table 2. Major changes obtained in haematology and coagulation measurements (group mean values ± standard deviation) in male and female rats treated intravenously for 4 weeks and 2 weeks of recovery period with 0 (vehicle = 0.9% saline solution), 5, 9 and 15 mg/kg/day of SET-M33 peptide. Hct haematocrit, Hb haemoglobin, RBC erythrocyte count, Retc reticulocyte count (absolute and relative), MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration, MCV mean corpuscular volume, WBC leucocyte count, total, HDW haemoglobin concentration distribution width, Lymphocytes, LUC large unstained cells, Plt platelet (thrombocyte) count, SPT prothrombin time, SAPT activated partial thromboplastin time. Significant differences between peptide vs. control groups were expressed at the 5% (* < 0.05) or 1% (** < 0.01) level. For statistical analysis, Dunnett, Shirley' Williams, Wilcoxon and t-tests were used.

Four-week toxicity study with 2-week recovery period in rats. The purpose of this study was to evaluate the toxic effects of SET-M33 when administered intravenously to Sprague Dawley rats for 4 weeks. Recovery was evaluated during a 14-day drug-free period. The peptide was administered once a day at three dose levels at 5, 9 and 15 mg/kg/day, and the control group was treated with the vehicle (ten males and ten females per group which were sacrificed after 4 weeks for clinical and necropsy analyses; five males and five females for the control group and 15 mg/kg/day groups which were sacrificed after the recovery period). The higher dose was selected on the basis of the DRF study (above) in which evident toxicity was recorded at the dose of 20 mg/kg. The efficacy dose in mouse is 5 mg/kg. 24 The lowest dose of this test is approximately twice the equivalent efficacy dose in rat (using body surface area) and the intermediate dose is approximately the geometric mean of the other two.

Mortality was only recorded at 15 mg/kg/day. One male died on day 8 and one male and one female were euthanized for animal welfare reasons on days 6 and 10 of the recovery period, respectively, after showing various clinical signs (hunched back, abnormal gait and pallor) and weight loss of 14% and 11%, respectively. SET-M33-related effects consisting of decreased motor activity, irregular breathing, piloerection, closed or partially closed eyelids, abnormal gait and hunched back were recorded in animals treated at 15 mg/kg/day just after administration for the whole treatment period (as in the DRF study above). From day 8 of recovery onwards, hunched back and pallor were observed in animals treated at 15 mg/kg/day. Among animals that had to be sacrificed for welfare reasons, abnormal gait, partially closed eyelids (the male) and decreased motor activity and piloerection (the female) were also recorded from day 3 onwards. Effects on body weight were observed mainly in males during the treatment period. During the recovery period, body-weight loss was observed mainly at doses of 9 and 15 mg/kg/day. A significant increase in white blood cells

Clinical laboratory investigations. Blood, coagulation, biochemistry and urinalysis parameters were evaluated at the end of treatment and at the end of recovery. A complete list of parameters can be found in Materials and Methods. A dose-related decrease in red blood parameters (haematocrit, haemoglobin, red blood cells and reticulocyte count) was noted in males and females at all dose levels, but was most pronounced at 15 mg/kg/day. Differences in other parameters, such as mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and haemoglobin concentration distribution width (HDW) were also observed, mainly at doses of 9 and 15 mg/kg/day. A signiﬁcant increase in white blood cells

AUC and Cmax values for the low vs. high dose were close to the theoretical ratio of 2 (values: 2.2 for AUCt and 2.4 to 2.7 for Cmax). On day 7, mean AUCt and Cmax values for the low vs. high dose were higher than the theoretical ratio of 2 (values: 9.8–14.0 for AUCt and 2.8–7.2 for Cmax). At 20 mg/kg, exposure to SET-M33 was higher on day 7 than on day 1, whereas no accumulation or low accumulation was observed at 10 mg/kg. Mean SET-M33 exposures were comparable for males and females in all groups and on days 1 and 7 (data not shown).
Biochemistry week 4 of treatment—Group mean values

| Sex | Dose (mg/kg/day) | ALP (U/L) | gGT (U/L) | Urea (µmol/L) | Creatinine (µmol/L) | Na (mmol/L) | Cl (mmol/L) | Phos (mmol/L) | Total Prot (g/L) | Alb (g/L) | SG1 (Vol mL) | Prot g/L | U-Gluc (mmol/L) | U-Creat (µmol/L) |
|-----|-----------------|-----------|-----------|---------------|---------------------|-------------|-------------|--------------|----------------|-----------|---------------|---------|----------------|------------------|
| Male | 0               | 130 ± 13.7| 0 ± 0.0   | 6.8 ± 0.05    | 31 ± 3.1           | 130 ± 3.1   | 98 ± 0.0    | 2.3 ± 0.05   | 2.3 ± 0.05   | 58 ± 5.2 | 31 ± 3.1       | 0.2 ± 0.04 | 2.6 ± 0.16      | 28 ± 1.6          |
|     | 5               | 146 ± 14.8| 0 ± 0.2   | 5.9 ± 0.15    | 30 ± 3.0           | 134 ± 2.5   | 92 ± 0.0    | 2.3 ± 0.05   | 1.9 ± 0.05   | 56 ± 3.6 | 32 ± 2.0       | 0.0 ± 0.04 | 1.0 ± 0.15      | 47 ± 1.5          |
|     | 9               | 187 ± 28.8| 0 ± 0.3   | 11.6 ± 0.3    | 35 ± 3.0           | 134 ± 2.4   | 105 ± 2.2   | 2.1 ± 0.06   | 2.2 ± 0.06   | 52 ± 3.3 | 37 ± 4.4       | 1.0 ± 0.20 | 2.5 ± 0.24      | 72 ± 1.6          |

**Table 3.** Major changes in biochemical and urinary parameters (group mean values ± standard deviation) in male and female rats treated intravenously for 4 weeks, followed by a 2-week recovery period with 0 (vehicle = 0.9% saline solution), 5, 9 and 15 mg/kg/day of SET-M33 peptide. ALP alkaline phosphatase, gGT gamma-glutamyl-transferase, Creat Creatinine, Na sodium, Cl chloride, Ca calcium, Phos inorganic phosphorus, Total Prot total protein, Alb albumin, SG1 specific gravity, Vol volume, Prot protein, U-Gluc glucose. Significant differences between peptide vs. control groups were expressed at the 5% (*p < 0.05) or 1% (**p < 0.01) level. For statistical analysis the Dunnett, Shirley, Williams, Wilcoxon and t tests were used.

(NBC), mainly due to an increase in lymphocyte and large unstained cell (LUC) count was recorded at 15 mg/kg/day, in both sexes. Platelet count was significantly higher than in the control group at all doses in males and females. Differences were dose-related. A slight increase in prothrombin time (SPT) and a decrease in activated partial thromboplastin time (SAPT) were recorded in both sexes at 9 and 15 mg/kg/day (Table 2). The effects on red blood cell parameters were not reversible after 2 weeks of recovery, whereas the effect on lymphocyte count seemed to be reversible, as no significant differences compared to control were recorded in males or females (Table 2). A significant dose-related increase in alkaline phosphatase was recorded at all dose levels in animals treated at 9 and 15 mg/kg/day. Increased gamma-glutamyl-transferase (gGT) was also observed in females at 9 and 15 mg/kg/day. A sharp dose-related effect on urea and creatinine was recorded at all doses, being statistically significant in all cases except in females at 9 mg/kg/day. Urea levels were more than 4 times control group values at 15 mg/kg/day. Creatinine values were more than 6 times control group values at 15 mg/kg/day. A significant decrease in albumin levels (and in total protein levels in males) was observed at 15 mg/kg/day (Table 3). Differences in alkaline phosphatase, gGT (mainly in females), bilirubin, urea and creatinine values had not reversed after 2 weeks of recovery.

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The purpose of this study was to conduct DRF for SET-M33 administered by short intravenous infusion (30 min) to beagle dogs at 4.0 and 8.0 mg/kg/day (7-day treatment period).
Figure 1. Effects of intravenous injection of SET-M33 at 0 (vehicle), 5, 9 and 15 mg/kg on respiratory rate (breaths/min ± se), tidal volume (mL ± se) and minute volume (mL/min ± se) in rats. Baclofen at 15 mg/day was used as positive control. se standard error of mean. Symbols (triangle, circle, rhombus or square) indicate a group mean respiratory parameter. The bar represents the standard error. The graphs were obtained using GraphPad Prism for Windows version 5.03, GraphPad Software, San Diego, California USA, www.graphpad.com. The groups treated with SET-M33 peptide were compared to vehicle using Williams’ test. The comparison between positive control and vehicle were made using two-tailed t test based on the error mean square from the analysis of covariance.
concentration (tmax) was after the end of infusion for both periods (days 1 and 7) and sexes, i.e. 30 min post-dose, suggesting one male on 8 mg/kg/day showed quantifiable concentrations at day 7 up to 24.5 h after the start of infusion. At doses of 4 and 8 mg/kg/day showed quantifiable concentrations until 3.5 h after starting the infusion. In addition, drug dose dependency, accumulation ratio and sex-related differences. Overall, on day 1, SET-M33 profiles observed coherently with intravenous administration. On day 1 and 7, mean AUC and Cmax values for the low vs. high dose were close to the theoretical ratio of 2. No accumulation or low accumulation was observed at 4 and 8 mg/kg after 7 days. Mean SET-M33 exposures were comparable for males and females in all groups on days 1 and 7, with a male/female ratios ranging from 0.3 to 1.3 for Cmax and from 0.4 to 1.3 for AUCt (data not shown).

Four-week toxicity study with 4-week recovery period in dogs. Beagle dogs (5/sex/group) were dosed by intravenous infusion once daily for 1 h with 0 (0.9% sodium chloride for injection), 0.5, 1.5 or 4.0 mg/kg/day SET-M33 for four consecutive weeks. The doses were selected in the dog DRF study (above). The dose of 4 mg/kg/day was selected as the highest dose without macroscopic side effects in the DRF study, and was used to evaluate renal toxicity and its reversibility. The dose of 0.5 mg/kg/day was selected as the lowest dose, about one order of magnitude less than the highest dose. The dose of 1.5 mg/kg/day was selected as an approximately median mid-dose. On completion of 4 weeks of infusion, three animals/sex/group were euthanized and necropsied and the remaining two animals/sex/group were held for a 4-week drug free recovery period after which they were euthanized and necropsied. Blood samples were obtained from all animals on days 1 and 28 for toxicokinetic analysis.

All animals survived until their scheduled termination and there were no noteworthy peptide-related clinical signs.

Clinical laboratory investigations. Blood, coagulation, biochemical and urine parameters were evaluated pre-test and on completion of treatment. The complete list of parameters evaluated is reported in Materials and Methods. Increased urea and creatinine levels were recorded in males and females on 8 mg/kg/day (67% and 74% higher than pre-test values for males, and 26% and 21% higher than pre-test values for females, respectively). No noteworthy effects on blood and coagulation parameters were recorded. Group mean values of significant changes are reported in Table 5.

Necropsy. A full necropsy was performed in all animals. Organs were collected and weighed. No noteworthy changes in organ weight were reported for the doses tested, except for lower weight of the thymus in males on doses of 4 and 8 mg/kg/day (66 and 76% less than control, respectively).

Bioanalytic and toxicokinetic study. Concentrations of SET-M33 in dog plasma samples and the main toxicokinetic parameters were determined. The exposure parameters (AUCt and Cmax) were compared to evaluate dose-dependency, accumulation ratio and sex-related differences. Overall, on day 1, SET-M33 profiles observed at doses of 4 and 8 mg/kg/day showed quantifiable concentrations until 3.5 h after starting the infusion. In addition, one male on 8 mg/kg/day showed quantifiable concentrations at day 7 up to 24.5 h after the start of infusion. Mean SET-M33 plasma levels increased in parallel in males and females. Mean time to maximum SET-M33 concentration (tmax) was after the end of infusion for both periods (days 1 and 7) and sexes, i.e. 30 min post-dose, coherently with intravenous administration. On day 1 and 7, mean AUCt and Cmax values for the low vs. high dose were close to the theoretical ratio of 2. No accumulation or low accumulation was observed at 4 and 8 mg/kg after 7 days. Mean SET-M33 exposures were comparable for males and females in all groups on days 1 and 7, with a male/female ratios ranging from 0.3 to 1.3 for Cmax and from 0.4 to 1.3 for AUCt (data not shown).

Clinical laboratory investigations. Blood, coagulation, biochemical and urine parameters were evaluated. A complete list of parameters is reported in Materials and Methods. There were no SET-M33-related blood or coagulation changes at the end of treatment or in the 4-week recovery period. SET-M33-related biochemical changes at all doses in both sexes included increases in blood urea nitrogen (BUN, 25–64% higher than pre-test) and creatinine (14–50% higher than pre-test) in individual animals (data not show). The magnitude of these

| Sex  | Dose (mg/kg/day) | Urea (nmol/L)          | Creatinine (µmol/L) |
|------|------------------|------------------------|---------------------|
|      |                  | Pre-dose | End of treatment | Pre-dose | End of treatment |
| Male | 0                | 5.15     | 4.58    | 79       | 58               |
|      | 4.0              | 4.31 ± 0.849 | 4.92 ± 0.035 | 84 ± 18.4 | 77 ± 2.8       |
|      | 8.0              | 4.26 ± 0.064 | 7.64 ± 0.191 | 66 ± 4.2  | 115 ± 25.5     |
| Female | 0           | 4.57     | 5.2     | 58       | 58               |
|      | 4.0             | 3.76 ± 0.156 | 4.76 ± 0.021 | 69 ± 17.7 | 79 ± 2.8       |
|      | 8.0             | 6.29 ± 2.15 | 7.94 ± 0.099 | 85 ± 23.3 | 103 ± 0        |

**Table 5.** Major differences of urea and creatinine in clinical biochemistry values (group mean values ± standard deviation), in beagle dogs treated with intravenous 30 min-short infusion of SET-M33 at 0 (vehicle = 0.9% saline solution), 4.0 and 8.0 mg/kg/day (7-day treatment period). Statistical analysis was not performed due to the small number of animals.
changes was generally similar across all dose groups. These increases were correlated with minimal renal tubule degeneration/regeneration at 1.5 mg/kg/day and 4 mg/kg/day in males and at 4.0 mg/kg/day in females and minimal interstitial cell infiltrate in both sexes at a dose of 4.0 mg/kg/day. There were no SET-M33-related biochemical changes at any dose after 4-week recovery, indicating complete recovery of the changes detected at the end of dosing. There were no SET-M33-related urine changes at 0.5 or 1.5 mg/kg/day. There were decreases in urine specific gravity and urine creatinine concentrations with respect to individual pre-test values in individual animals at 4.0 mg/kg/day. These changes were more prominent in females and suggest more dilute urine, possibly associated with a lower concentrating ability of the kidneys. There were no SET-M33-related urine changes at any dose after the 4-week recovery period.

Necroscopy. Higher SET-M33-related kidney weights (absolute and relative to body and brain weight) were observed in both sexes at a dose of 4.0 mg/kg/day. These higher weights were correlated microscopically with minimal tubule degeneration/regeneration and minimal interstitial cell infiltrates. There were no other SET-M33-related organ weight changes (Table 6). There were no organ weight changes at recovery sacrifice, or macroscopic findings at terminal and recovery necroscopy. SET-M33-related microscopic findings were observed in kidneys at doses of 1.5 mg/kg/day and 4 mg/kg/day at infusion sites at all doses. The kidney findings

| Male | 0 mg/kg/day | 0.5 mg/kg/day | 1.5 mg/kg/day | 4.0 mg/kg/day |
|------|-------------|---------------|---------------|---------------|
| 0 mg/kg/day | 0 | 1 | 0 | 0 |
| 0.5 mg/kg/day | 0 | 1 | 0 | 0 |
| 1.5 mg/kg/day | 0 | 1 | 0 | 0 |
| 4.0 mg/kg/day | 0 | 1 | 0 | 0 |

| Female | 0 mg/kg/day | 0.5 mg/kg/day | 1.5 mg/kg/day | 4.0 mg/kg/day |
|--------|-------------|---------------|---------------|---------------|
| 0 mg/kg/day | 0 | 1 | 0 | 0 |
| 0.5 mg/kg/day | 0 | 1 | 0 | 0 |
| 1.5 mg/kg/day | 0 | 1 | 0 | 0 |
| 4.0 mg/kg/day | 0 | 1 | 0 | 0 |

Table 6. SET-M33-related changes in beagle dogs dosed with the peptide at 0 (vehicle = 0.9% saline solution), 0.5, 1.5 and 4.0 mg/kg/day for 28 days and after 4-week recovery. For kidney weight changes, statistical analysis was not performed due to the small number of animals.
included minimal tubule degeneration/regeneration at 1.5 mg/kg/day and 4 mg/kg/day in males, and at 4.0 mg/kg/day in females, and minimal interstitial cell infiltrate in both sexes at a dose of 4.0 mg/kg/day. Degeneration featured vacuolation, cell sloughing and/or tinctorial change, and regeneration was characterized by increased basophilia, nuclear crowding and/or increased mitoses of cortical tubule epithelial cells. Regenerative changes were typically more pronounced than degenerative changes. SET-M33-related microscopic findings in the kidney were correlated with higher kidney weights (absolute and relative to body and brain weight) and elevated BUN and creatinine in both sexes at 4.0 mg/kg/day. SET-M33-related findings were observed at all four infusion sites (different site each week) in both sexes at all doses and included vascular thrombi (minimal to high), vascular/perivascular inflammation (minimal to moderate), hypertrophy/hyperplasia of the tunica intima or tunica media (minimal to slight), and/or vascular/perivascular haemorrhage (minimal to moderate). The other SET-M33-related microscopic findings in kidney and at infusion sites were considered non-adverse due to the magnitude of the changes (minimal to moderate) and/or a lack of correlations suggesting functional impairment. After the 4-week recovery period, kidney interstitial infiltrates cleared completely and tubule degeneration/regeneration resolved almost completely. At the infusion sites, there was complete recovery from vascular/perivascular inflammation and tunica intima hypertrophy/hyperplasia with partial recovery from vascular thrombi and hypertrophy/hyperplasia of the tunica media. Minimal regeneration of cortical tubule epithelial cells, observed in the kidneys of 1 female at a dose of 4.0 mg/kg/day, was considered to be due to a repair process, and a slight thrombus observed at the infusion site (saphenous vein) of another female was also in line with ongoing repair. In addition, slight tunica media hypertrophy/hyperplasia of the left cephalic vein was observed in one male (Table 6). Slight tunica media hypertrophy/hyperplasia of the left cephalic vein was observed in 1 male at a dose of 4.0 mg/kg/day.

As a final result of this toxicological study, the no-observed-adverse-effect-level (NOAEL) of SET-M33 was determined to be 0.5 mg/kg/day.

Bioanalytics and toxicokinetics in rats and dogs. Unlike similar tests reported in DRF studies, here SET-M33 was administered for a prolonged period (4 weeks instead of 1 week) and at doses previously selected by DRF. In order to better relate the toxicokinetics data (presented below) with SET-M33 activity we report, as examples, the following SET-M33 MIC values: MIC50 and MIC90 for P. aeruginosa are 1.4 µM; MIC50 and MIC90 for K. pneumoniae are 1.4 µM and 2.8 µM, respectively24.

RATS. SET-M33 was administered intravenously as a slow bolus to Sprague Dawley rats once daily for 4 weeks at 0 (vehicle only), 5, 9 and 15 mg/kg/day (control group 3 animals/sex, treated groups 6 animals/sex). Blood samples were taken on day 1 and day 28. The SET-M33 profiles showed quantifiable concentrations until 1 h post-dose at days 1 and 28. Mean plasma SET-M33 exposures were higher for females than males at day 28, while no differences were observed at day 1. Mean plasma SET-M33 exposure increased in parallel with dose in males and females. Males and (especially) females showed higher exposure levels at day 28 than day 1 (Fig. 2). Mean time to maximum plasma SET-M33 concentration (tmax) was 5 min post-dose for both times and sexes as expected for intravenous bolus administration. On day 1, mean AUC0-t and Cmax values for the low vs. intermediate and high doses were close to the theoretical ratios of 1.8 and 3 (2 and 2.9–3.2 for AUC0-t,1.7–2 and 2.7 for Cmax). On day 28, female intermediate dose AUC0-t, ratio was 1.7 times the theoretical ratio. The results suggest accumulation of SET-M33 in all dose groups in males and especially in females. This accumulation does not seem due to the repeated dose, since no steady state was reached (Table 7).

Dogs. Plasma concentrations and the main toxicokinetic parameters of SET-M33 in plasma samples were determined in beagle dogs after intravenous administration of SET-M33 by daily 1-h-infusion at 0.5, 1.5 or 4.0 mg/kg/day or only vehicle for 4 weeks (5 animals/group/sex). Blood samples were taken on day 1 and day 28. Mean plasma concentration–time profiles showed higher values for all doses at day 28 (Fig. 3). Maximum plasma concentrations (Cmax) of SET-M33, their times of occurrence (Tmax) and the areas under the plasma SET-M33 concentration–time curves within a 24-h dosing interval (AUC0-24) on day 1 and day 28 are shown in Table 8, where the mean Cmax and AUC0-24 for the group are shown with standard deviations in brackets. The time when the maximum plasma concentration occurred (Tmax) was at the end of the 1-h infusion in all animals, as expected for this route of administration. Plasma concentrations of SET-M33 at 24 h post-dose were below the limit of quantification (< 20.0 ng/ml) in all animals at all dose levels on day 1 and day 28 (not shown).

The systemic exposure (Cmax and AUC0-24) of dogs to SET-M33 increased with increasing dose over the dose range 0.5–4.0 mg/kg/day on day 1 and day 28. Excluding Cmax values in males, the Cmax and AUC0-24 values at the highest dose (4.0 mg/kg/day) were approximately 2.1 times higher than those predicted in the case of a linear relationship (not shown). The Cmax and AUC0-24 Values of SET-M33 in female dogs were similar to the indices of exposure in males at the two lower dose levels, but were approximately 1.6 times higher than those of males at the highest dose level (Fig. 4). After repeated doses (day 28), Cmax and AUC0-24 Values of SET-M33 were generally higher than those after a single dose (day 1) (Fig. 4). The accumulation ratios, based on Cmax and AUC0-24, values, were generally greater than one, indicating that systemic exposure to SET-M33 was higher after repeated administrations than after a single dose (not shown). However, since plasma concentrations of SET-M33 were below the limit of quantification 24 h post-dose in all animals, these results indicate that SET-M33 has time-dependent kinetics.
Discussion

The standard procedures indicated by regulatory agencies specify that before a new therapeutic entity can be given to humans, developers must first test it thoroughly in animals for safety and efficacy. The main aims of pre-clinical studies are roughly to determine: (i) the efficacy and toxicity of the compound; (ii) its pharmacokinetics; (iii) the formulation for appropriate delivery in humans. Pre-clinical toxicological studies must be...
Figure 3. Mean plasma concentrations of SET-M33 on day 1 (A) and (C) and day 28 (B) and (D) of 4 weeks of daily intravenous (infusion) administration to male (A) and (B) and female (C) and (D) dogs at 0.5, 1.5 and 4.0 mg/kg/day. Symbols (circle, triangle or square) indicate a group mean value. Bars represent standard deviations. The graphs were plotted using GraphPad Prism for Windows version 5.03, GraphPad Software, San Diego, California USA, www.graphpad.com.

Table 8. Pharmacokinetic parameters of SET-M33 on day 1 and day 28 of 4 weeks of daily intravenous (infusion) administration to male and female beagle dogs at 0.5, 1.5 and 4.0 mg/kg/day (group mean values ± standard deviation of $C_{\text{max}}$ and $\text{AUC}_{0-24}$). $\text{AUC}_{0-24}$ area under the plasma concentration–time curve in 24-h dosing intervals, $C_{\text{max}}$ maximum plasma concentrations, EOI end of infusion, $t_{\text{last}}$ time point of the last quantifiable plasma concentration, $t_{\text{max}}$ time at which $C_{\text{max}}$ occurred. Statistical analysis is not applicable to these experiments.
Conducted in two animal species, including a rodent and a non-rodent, before proceeding to the clinical phases of development. In the preclinical phase, the ultimate goal is to translate the animal model responses into an understanding of the risk for human subjects. Toxicity testing in animals is therefore valuable for lead compound characterization and further decisions about the direction of development.

The peptide SET-M33 is a synthetic molecule under study for the development of a new antibacterial drug. SET-M33 and some of its back-up molecules have already been reported for antibacterial efficacy in different infections and inflammation models in vivo and ex vivo, including sepsis, pneumonia, and skin infections. As a novel drug to administer intravenously, SET-M33 has entered a preclinical development phase that includes scale up of production, adsorption, excretion, and finally toxicity, the subject of the present report.

In the present study, SET-M33 showed a grade of toxicity, which when combined with the efficacy experiments already reported, suggests that it will have a favourable therapeutic index.

When tested in rats, even at highest dose used, SET-M33 did not have any neurological effects as assessed by Irwin tests of behavioural and physiological states, nor did it affect body temperature, locomotor activity or body weight. Likewise, SET-M33 did not affect lung function in terms of respiratory rate, tidal volume and minute volume when compared with the vehicle-treated control and a baclofen-treated positive control.

In prolonged treatment (4-week daily administration and 2-week recovery period) of rats, clinical signs during the recovery period and loss of body weight recorded in some animals showed that animals treated with the high dose (15 mg/kg/day) were unable to recover. Mean plasma concentrations quantified on day 28 were higher than on day 1 in both sexes, but especially in females. In any case, because no quantifiable concentrations were detected in the day 28 pre-dose and 24-h post-dose samples, the increased drug exposure in females at day 28 is more likely related to a defect in drug elimination than to accumulation of SET-M33.

Systemic exposure of dogs to SET-M33 generally appeared to have nonlinear (dose-dependent) kinetics in the dose range 0.5 to 4.0 mg/kg/day on days 1 and 28 of the 4-week intravenous (infusion) study. Increasing the dose of SET-M33 above 0.5 mg/kg/day is likely to result in higher systemic exposure than would be predicted in the case of a linear relationship. However, the Cmax of SET-M33 in male dogs appeared to have linear (dose-independent) kinetics. In addition, the data also provided evidence that there were no differences in systemic exposure of male and female dogs to SET-M33 at the 0.5 and 1.5 mg/kg/day dose levels, but that at the highest dose (4.0 mg/kg/day), the systemic exposure of female dogs to SET-M33 was higher than that of males. Systemic exposure was higher after repeated intravenous administration (infusion) of SET-M33 than after a single dose and suggests that SET-M33 has time-dependent kinetics.

Daily intravenous infusion of the peptide at ≥ 0.5 mg/kg/day in beagle dogs did not produce any observable effect on heart function, as demonstrated by ECG parameters (heart rate, PR, QRS, QT and QTc intervals) in males and females (data not reported here).

In both animal species, daily administration of SET-M33 at the highest doses used in our tests caused renal effects, such as tubule degeneration/regeneration, elevated blood concentrations of urea and creatinine, high glucose (only in rats), all suggesting a functional deficit and identifying the kidneys as a possible target for toxic effects. This confirms the bio-distribution and excretion data obtained previously with radio-iodinate SET-M33, which showed an evident uptake of the peptide by kidneys and bladder after intravenous administration of the peptide to mice. The bioanalytical evaluation of blood parameters and the histological analysis of the present study did not suggest any possible toxic effects on other organs in rats and dogs.
Dogs remain the main non-rodent species used in preclinical drug development\(^6\). Determination of the NOAEL in these animals is an important part of non-clinical risk assessment\(^{30,31}\). The results obtained with SET-M33 in the 4-week toxicity study with 4-week recovery period in dogs, especially regarding the adverse findings of renal tubule degeneration/regeneration and moderate to marked vascular thrombi at infusion sites at doses ≥ 1.5 mg/kg/day, indicate that the NOAEL is below this latter dosage, presumably around 0.5 mg/kg/day. This dose will be a useful starting point for an eventual phase I clinical trial.

All the studies reported in this article were designed to comply with accepted pharmacological principles and the requirements of Europe, Japan and the USA\(^{30,31,42–49}\).

**Methods**

**SET-M33 peptide.** Peptide with a purity of 97.9% as declared by the producer (Polypeptide, Strasbourg) was used for all tests. The formulations were prepared under sterile conditions by dissolving the powder in the vehicle (0.9% sodium chloride) and then sterile filtering with a 0.22 micron PVDF filtration unit. Dose formulations of SET-M33 and 0.9% sodium chloride for injection were analysed to confirm that the prepared dose formulations were homogeneous and that the administered SET-M33 concentrations were appropriate under the study conditions. The analytical method validated at the Testing Facility involved dilution of SET-M33 dose formulation samples in 100% water followed by quantification using high performance liquid chromatography with ultraviolet detection (HPLC–UV).

**Animals.** *Rats.* Sprague Dawley rats were used for the toxicity studies. The animals were supplied by Envigo RMS S.L. All animals were 6–8 weeks old at the start of treatment and were allocated randomly to the treatment groups. The peptide was administered as an intravenous bolus in approximately 50 s via the lateral tail vein using a graduated syringe and a 24G (0.55 × 25 mm) needle. The administration volume was 5 mL/kg body weight.

**Dogs.** Naïve beagle dogs were used for the toxicity studies. The animals were supplied by Marshall US. They were 9–10 months old at the start of treatment with a body-weight range of 9.1–10 kg for the DRF study. They were 6–7 months old at the start of treatment with a body-weight range of 6.6–8.6 kg for males and 5.1–6.5 kg for females for the 4-week toxicity study. The method of administration was an intravenous short infusion with an infusion pump, alternately either into the cephalic or saphenous veins using sterile disposable cannulas and syringes. The volume of the dose of SET-M33 and control in two studies was 2, 5 and 5 mL/Kg body weight, respectively.

All experimental protocols were approved by licensing committees from the institutions where the experiments were carried out. All animal experiments were performed in collaboration with the following CROs: Covance CRS LLC (now Labcorp Drug Development), Huntingdon, Cambridgeshire UK and Somerset, New Jersey USA; AnaPath Research S.A.U., Castellar de Vallès, Barcelona, Spain.

All experimental procedures were carried out in accordance with the following guidelines and regulations: the United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012; USA Animal Welfare Act Regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, Volume 54, N. 168, August 31, 1989, pp. 36,112–36,163 effective October 30, 1989 and 9 CFR Part 3 Animal Welfare Standards; Final Rule, Federal Register, Volume 56, N. 32, February 15, 1991, pp. 6426–6505 effective March 18, 1991; Decree (Decree) 214/1997 of 30 July, Ministry of Agriculture, Livestock and Fishing of the Autonomous Government of Catalonia; Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes; Law 5/1995 of 21 June on the protection of animals used for experimentation and other scientific purposes (DOGC 2073, 10.7.1995), Autonomous Government of Catalonia; Law 6/2013 of 11 June, amending Law 32/2007 of 7 November on the care of animals during their exploitation, transport, experimentation and sacrifice, Spain; Real Decreto (Royal Decree) 53/2013 of 1 February 2013, Spain.

All methods are reported in accordance with ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments\(^6\)).

The number of animals used was the minimum consistent with scientific integrity and regulatory acceptability, considering the welfare of individual animals in relation to the number and extent of procedures to be carried out on each animal.

**Dose range finding (DRF) in rats.** A viability/mortality check was recorded at least twice daily. Detailed observation of clinical signs was made daily during dosing.

**Haematology.** Blood samples were drawn from the retro-orbital plexus of all animals under light isoflurane anaesthesia. Blood was collected into tubes containing EDTA-K\(_3\) as anticoagulant. The following parameters were determined using an ADVIA 120 haematology analyser (Siemens Healthcare): Red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), reticulocyte count (absolute and relative) (Retic), platelet count (Plt), total leukocyte count (WBC), neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E), basophils (B), large unstained cells (LUC).

**Clinical biochemistry.** Blood samples were collected into lithium heparin tubes. The plasma was analysed for the following parameters with a Cobas 6000 analyzer (Roche): glucose (Gluc), urea (Urea), creatinine (Creat), bilirubin, total (Bili), cholesterol, total (Chol), triglycerides (Trig), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), gamma-glutamyl-transferase (gGT), calcium (Ca), inorganic
phosphorus (Phos), sodium (Na), potassium (K), chloride (Cl), total protein (total Prot), protein electrophoregram, albumin (Alb), globulin (calculated from the total protein and Alb%) (Glob), album/globulin ratio (A/G ratio).

**Urinalysis.** Urine was collected in specimen vials using a metabolism cage. The following parameters were determined using a Cobas u 411 semi-automated test strip analyser (Roche): specific gravity (SG1), volume (Vol), colour (Col), appearance (App), pH, nitrite (Nite), protein (Prot), glucose (U-Gluc), ketones (Keto), urobilinogen (Urob), bilirubin (Bill), erythrocytes (U-RBC), leukocytes (U-WBC).

**Necroscopy.** Necroscopy was performed after the end of treatment in Phases I and II. Animals were sacrificed by intraperitoneal injection of sodium pentobarbital and immediately exsanguinated. Gross necropsy examination of the cranial, thoracic, and abdominal cavities, major organs and injection site was performed on Phase I animals. A full necropsy was performed on Phase II animals, including examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavity organs in situ and after evisceration.

**Bioanalytic and toxicokinetic study.** Blood samples were collected from the retro-orbital plexus of animals under light isoflurane anaesthesia. Blood samples were taken from three males and three females at each extraction time. Sampling times were as follows: Day 1: 5, 15, 30 and 60 min after administration. Day 7: pre-dose and 5, 15, 30, 60 min and 24 h after administration. Control animals were bled only once on days 1 and 7 of treatment at 30 min after treatment. Each blood sample was collected into a polypropylene test tube containing lithium heparin as anticoagulant and kept in an ice bath until centrifuging (1600 g for 10 min at 2–8 °C). The plasma from each sample was transferred to a fresh polypropylene test tube, immediately frozen in dry ice and stored at −20 °C ± 5.

**Four-week toxicity study with 2-week recovery period in rats.** Viability/mortality was monitored twice daily throughout the study. Detailed clinical signs were evaluated once at pre-test, once/twice daily during the treatment period and on days 1, 8, 10 to 14 of the recovery period. Body weight was monitored once at pre-test, twice weekly during treatment and recovery periods, at termination of treatment and before sacrifice (scheduled animals fasted).

**Haematology.** Blood samples were extracted from the retro-orbital plexus under light isoflurane anaesthesia. The sample were collected into tubes containing EDTA-K$_3$ anticoagulant. The following parameters were determined using an Advia 120 haematology analyser (Siemens Healthcare): red blood cell count (RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscle volume (MCV), red cell volume distribution width (RDW), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin concentration distribution width (HDW), reticulocyte count (Retic), platelet (thrombocyte) count (Plt), leukocyte count, total (WBC), neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E), basophils (B), Irge unstained cells (LUC).

**Coagulation.** Blood samples were collected into 3.2% sodium citrate tubes to obtain the plasma. The following parameters were determined using a STA COMPACT Automatic Coagulometer: prothrombin time (SPT), activated partial thromboplastin time (APTT).

**Clinical biochemistry.** Blood samples were collected into lithium heparin tubes. The following parameters were determined using a Cobas 6000 Analyzer (Roche): glucose (Gluc), urea (Urea), creatinine (Creat), bilirubin, total (Bill), cholesterol, total (Chol), triglycerides (Trig), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), gamma-glutamyl-transferase (gGT), calcium (Ca), inorganic phosphorus (Phos), sodium (Na), potassium (K), chloride (Cl), albumin (Alb), globulin (calculated from total protein and Alb%) (Glob), total protein (Total Prot), album/globulin ratio (A/G ratio).

**Urinalysis.** Urine was collected overnight into specimen vials (animals were placed in metabolism cages at the end of the working day preceding the day of urine collection). The following parameters were determined using a Cobas u 411 semi-automated test strip analyser (Roche): specific gravity (SG1), protein (Prot), ketones (Keto), volume (Vol), creatinine (U-Creat), bilirubin (Bill), colour (Col), glucose (U-Gluc), erythrocytes (U-RBC), appearance (App), nitrite (Nite), pH, leukocytes (U-WBC).

**Necroscopy.** All animals underwent necropsy. Descriptions of all macroscopic abnormalities were recorded. Samples of tissues and organs were collected from all animals, weighed and fixed in neutral phosphate-buffered 4% formaldehyde solution (10% formalin). All organ and tissue samples to be examined were processed, embedded, cut and stained with haematoxylin and eosin.

**Neurological toxicity in rats.** Evaluation of SET-M33 neurological toxicity was based on the method described by Irwin$^{32,33}$. On the day prior to dosing, detailed subjective observation of all animals was made to assess the neurobehavioral and physiological state of untreated rats. After subjective observation, rectal temperature was measured and spontaneous locomotor activity was assessed. On the day of dosing, the animals were treated and then returned to their home cages. Detailed subjective observation of the rats was then repeated 5, 30, 90 and 240 min after dosing. A further observation was made 24 h after dosing. During these observations,
the following parameters were systematically evaluated for each animal using a standard procedure: lethality,
restlessness, apathy, writhing, fighting, stereotyped behaviour, tremor, twitches, convulsions, exophthalmos,
abnormal respiration, alertness, startle response, loss of fighting reflex, abnormal body carriage, abnormal gait,
Straub tail, piloerection, pupil diameter, light-pupil response, touch response, fearfulness, pinna reflex, corneal
reflex, catalepsy, passivity, aggressiveness, body tone, grip strength, cutaneous blood flow, cyanosis, ptosis, lac-
rimation, salivation, pain response, motility impairment, grooming, diarrhoea, vocalization, increased urina-
tion. Normal attributes of animals (e.g. alertness, body tone etc.) were subjectively scored as 4; enhancement or
depression of these attributes by SET-M33 was scored with higher or lower integers, respectively. Attributes nor-
maIy absent in animals (e.g. abnormal gait, abnormal respiration, tremors etc.) were subjectively scored from
0 (normal) to 8. At the end of each observation period, the rectal temperature of each animal was measured.
Finally, spontaneous locomotor activity was analysed. Each animal was placed in a suitable arena and locomotor
activity measured in terms of number of squares crossed in a 2-min period. Animals were inspected daily from
day 3 to day 7 for appearance of any delayed effects. After the day 7 inspection, the animals were killed humanely
by a rising concentration of carbon dioxide. Death was confirmed by dislocation of the neck.

Evaluation of respiratory function in rats. Whole body, bias flow plethysmography equipment was
used1. Respiratory parameters (respiratory rate, tidal volume and minute volume) were derived from the
changes in pressure associated with the warming and humidification of the air breathed in by the animal. This
was monitored by specific probes located in the plethysmograph chambers. Bias flow (room air) was set at
approximately 2.5 L/min. On a day prior to the first day of dosing, all animals were habituated to the plethys-
mographs for approximately 2 h. The study was run over 4 days and 10 animals (two from each group) were
examined each day. On the day of dosing, the animals were placed in the plethysmographs for pre-dose record-
ing (session 1) of respiratory parameters for 60 min. Groups of 8 rats were then removed from the chambers
and dosed by intravenous bolus injection with vehicle, SET-M33 or positive control. Immediately after dosing,
the rats were placed in the whole body plethysmographs, where they could move about freely, to continuously
record respiratory parameters for 4 h post-dose (undisturbed recording). Immediately after the last post-dose
recording, the animals were killed humanely by a rising concentration of carbon dioxide. Death was confirmed
by dislocation of the neck. Respiratory parameters were reported at the following time points: 0, 30, 60, 90,
120, 150, 180, 210 and 240 min. Time 0 coincided with the mean value of the last 20 min of data recorded in
the 60-min pre-dose period. All other time points were the mean of 10-min recordings (an average of every 10
breaths) around each time point. Each post-dose time point was analysed separately by analysis of covariance.
Factors in the model were group and day of data collection, with pre-dose values as covariate.

Dose range finding (DRF) in dogs. Viability/mortality were recorded at least twice daily. On treatment
days, all animals were observed for signs of toxic or pharmacological effects prior to, during and immediately
after administration and 1–2 h post-dose. Injection sites were examined daily during dosing. For clinical inves-
tigation, blood obtained via jugular venepuncture from anaesthetized dogs was used to analyse blood, coagulation
and biochemical parameters.

Haematology. A blood sample was collected into tubes containing EDTA-K3 as anticoagulant and analysed
for the following parameters using Advia 120 haematology analyser (Siemens Healthcare): red blood cell count
(RBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglo-
bin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (Plt), total leukocyte count
(WBC), reticulocyte count (Retic), neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E) basophils
(B), large unstained cells (LUC).

Clinical biochemistry. A blood sample was collected in lithium heparin tubes. The following parameters were
determined using Cobas 6000 analyzer (Roche): albumine (Alb), glucose (Gluc), urea (Urea), creatinine (Creat),
bilirubin (Bili), cholesterol (Chol), aspartate aminotransferase (AST), alanine aminotransferase (ALT), sodium
(Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (Phos), total protein (Total Prot), pro-
tein electrophoretogram.

Urinalysis. Urine obtained via a 16-h overnight collection period was analysed. Urine was collected into ice-
chilled containers overnight from pans placed beneath each animal's cage. Urine samples were analysed for the
following parameters using Multistix reagent strips, interpreted using a Siemens Clinitek Advantus: specific
gravity (SG1), colour (Col), pH, protein (Prot), glucose (U-Gluc), ketones (Keto), urobilinogen (Urob), bilirubin
(Bili).

Necropsy. All animals were sacrificed at the end of the treatment period by intravenous injection of sodium
pentobarbital. Organs were collected and weighed.

Bioanalytic and toxicokinetic study. Blood samples were taken by direct venepuncture of the jugular vein on
days 1 and 7 to determine plasma levels of SET-M33. Sampling times were as follows: Day 1: at 0, 5, 15, 30, 60 and
180 min. Day 7: at 0, 5, 15, 30, 60, 180 min and 24 h after administration. Control animals were only bled once on
days 1 and 7 of treatment, 1 h after administration. Blood samples were collected into lithium heparin test tubes
and kept in an ice bath until centrifuging (1600 g for 10 min at 2–8 °C). The plasma obtained from each sample
was transferred to a fresh polypropylene test tubes, immediately frozen in dry ice and stored at −20 °C ± 5.
Four-week study toxicity with 4-week recovery period in dogs. Animals were observed daily for mortality and signs of severe toxic or pharmacological effects. On treatment days, all animals were observed for signs of toxic or pharmacological effects prior to, during and after administration and 1–2 h post-dose. Blood obtained via jugular venepuncture from anaesthetized dogs was used to analyse blood, coagulation and clinical chemistry parameters for 5 animals/sex/group pre-test and at termination of dosing and from 2 animals/sex/group at the end of recovery.

Haematology. Blood samples were collected into tubes containing K₂EDTA anticoagulant and analysed for the following using ADVIA 120 Haematology Analyser (Siemens): haemoglobin (Hb), haematocrit (Hct), red blood cell count (RBC), platelet count (Plt), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), total white blood cell count (WBC), reticulocyte count (Retic), neutrophils (N), lymphocytes (L), eosinophils (E), basophils (B), monocytes (M), large unstained cells (LUC).

Coagulation. Blood samples were collected into tubes containing sodium citrate anticoagulant and analysed for the following using a Diagnostica Stago Products STA Compact MAX mechanical clot detection system: prothrombin time (SPT), activated partial thromboplastin time (APTT), fibrinogen (FIB).

Clinical biochemistry. Blood samples were collected into tubes with no anticoagulant, allowed to clot, centrifuged to obtain serum and analysed for the following using ADVIA 1800 Chemistry Analyzer (Siemens): aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), blood urea nitrogen (BUN), creatinine (Creat), glucose (Glu), cholesterol (Chol), triglycerides (Trig), total protein (Tot Prot), albumin (Alb), total bilirubin (Bili), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (Phos), gamma-glutamyl transferase (gGT).

Urinalysis. Urine obtained via a 16-h overnight collection period was analysed for all animals/sex/group pre-test, at study termination, and at the end of recovery. Urine was collected into ice-chilled containers overnight from pans placed beneath each animal’s cage. Urine samples were analysed for the following parameters using Multistix reagent strips, interpreted using a Siemens Clinitek Advantus: pH, protein (Prot), glucose (U-Gluc), ketones (Keto), bilirubin (Bili), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (Phos), gamma-glutamyl transferase (gGT).

Necropsy. Complete macroscopic examination was performed on all animals, including examination of the external surface and all orifices; the external surfaces of the brain and spinal cord; the organs and tissues of the cranial, thoracic, abdominal and pelvic cavities and neck; and the rest of the carcass for macroscopic morphological abnormalities. Necropsy was performed on up to 3 animals/sex/group after treatment for 4 weeks and on 2 animals/sex/group after a 4-week treatment-free recovery period. Organs and tissues were weighed, preserved and examined microscopically. Prior to weighing, organs were carefully dissected and properly trimmed to remove adipose and other contiguous tissue in a uniform manner. Organs were weighed as soon as possible after dissection in order to avoid drying. Paired organs were weighed together. Eyes, optic nerve and testes were initially placed in Modified Davidson’s solution and then kept in 10% neutral buffered formalin (NBF). Lungs and urinary bladder were infused with 10% NBF prior to immersion in a larger volume of the same fixative. All other tissues were preserved in 10% NBF.

Bioanalytics and toxicokinetics in rats and dogs. Rats. Blood samples were drawn from the retro-orbital sinus under light isoflurane anaesthesia for determination of plasma SET-M33 levels. The samples were taken on days 1 and 28 of treatment at the following times: control group: pre-dose and 30 min; 5, 9 and 15 mg/kg/day-group: pre-dose, 5, 15, 30, 60 min and 24 h after administration. Blood samples were collected into polypropylene test tubes containing lithium heparin anticoagulant and kept at room temperature for no longer than 60 min until centrifuging (1600 g for 10 min at 2–8 ºC). The plasma obtained from each sample was transferred to a fresh polypropylene test tube, immediately frozen in dry ice and stored at −20 ºC ± 5. Plasma concentrations of SET-M33 were measured by a previously validated LC–MS/MS method. Toxicokinetic parameters were determined for mean plasma concentrations at each dose level and time point, according to the validated method and with WinNonlin software, version 6.3, in the Phoenix Suite version 1.3 (Pharsight Corporation, Mountain View, CA, USA).

Dogs. On days 1 and 28, blood samples were obtained for toxicokinetic determinations from all animals at the following times: Day 1 at the end of infusion and 5, 10, 15, 30, 60 and 180 min later; 6 ± 15 min and 24 h ± 30 min post-dose; Day 28 at the end of infusion and 5, 10, 15, 30, 60 and 180 min later; 6 ± 15 min and 24 h ± 30 min post-dose. Blood samples were collected into lithium heparin test tubes and kept in an ice bath until centrifuging (1600 g for 10 min at 2–8 ºC). The plasma obtained from each sample was transferred to a fresh polypropylene test tubes, immediately frozen in dry ice and stored at −20 ºC ± 5. Bioanalytical samples were analysed by a validated liquid chromatographic mass spectrometric assay. Pharmacokinetic parameters were calculated using the computer program Phoenix WinNonlin version 6.3 (Certara USA, Inc).

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
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Author contributions
L.C. organized and wrote the article with the help of G.C., C.F., J.B. and L.B. participated in the experimental setup and interpretation of the data. S.V. and E.M. managed all activities in collaboration with CROs for animal procedures. A.P. coordinated the entire work.

Competing interests
The peptide SET-M33 is covered by a patent owned by the University of Siena and licensed to SetLance srl. Chiara Falciani, Alessandro Pini and Luisa Bracci are partner of SetLance. The rest of authors declare that they have no other competing financial interests.

Additional information
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