

In Vitro and *In Vivo* Activities of Antibiotic PM181104

Girish Mahajan,^{1#} Becky Thomas,¹ Rajashri Parab,¹ Zarine E. Patel,¹ Sandip Kuldharan,¹ Vijayaphanikumar Yemparala,¹ Prabhu Dutt Mishra,¹ Prafull Ranadive,¹ Lisette D'Souza,² Koteppa Pari,¹ and H. Sivaramkrishnan¹

Piramal Enterprises Limited, 1-Nirlon Complex, Goregaon, Mumbai, 400 063, India,¹ and Bioorganic Chemistry Laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa, India²

Corresponding author and Mailing address: Piramal Enterprises Limited, 1-Nirlon Complex, Goregaon, Mumbai, 400 063, India.

Phone: +91-22-30818834. Fax: +91-22-30818036. E-mail: girish.mahajan@piramal.com.

Abstract

The drug resistance has become a global threat and if not addressed we may enter in pre-antibiotic era. A way to overcome growing incidence of global antibiotic resistance issue is to introduce compounds belonging to classes that are new to the clinic. During our marine microbial screening for new antibiotics, our efforts led to the discovery of a novel thiazolyl cyclic-peptide antibiotic, PM181104. It was isolated and characterised from a marine sponge associated actinobacterium strain of the genus *Kocuria* (MTCC 5269). The compound exhibited a potent *in vitro* antibacterial activity against a broad range of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), other resistant strains of *S.aureus* and Vancomycin resistant enterococci (VRE). The minimum inhibitory concentration (MIC) values evaluated for the compound were found to be in nano-molar range. In its *in vivo* studies in Balb/c murine septicemia model the compound displayed an ED₁₀₀ value of 2.5 and 5.0 mg/kg against MRSA and 10.0 mg/kg against VRE. In the current report, the *in vitro* and *in vivo* studies of PM181104 are described.

Introduction

The last two decades have witnessed the dramatic rise in the prevalence of antibiotic resistance observed in bacterial infections. This posed a significant clinical problems and has become a growing threat to the public health worldwide. Infections caused by MRSA, Penicillin-resistant *Streptococcus pneumoniae* (PRSP) and Vancomycin resistant enterococci (VRE) are more of such concern, as many of these organisms have developed resistance to several classes of established antibiotics (1). The most significant problem faced in the clinical practice is the increase in incidences of MRSA infections. Numerous reports of emerging Vancomycin resistance in some of the MRSA isolates have also been recorded (2,3). The prospect for a major antibiotic emergency is well recapitulated by the Infectious Diseases Society of America (IDSA) (4,5) which reports that there are only a few potential drugs in clinical development that provide significant benefits over existing drugs and that target Gram negative, hospital-based infections. Linezolid, a well known antibiotic belonging to oxazolidinone class of compounds, and daptomycin, a new cyclic lipopeptide are currently being considered as the drugs of choice for the treatment of MRSA infections. However, due to rising incidences of resistance to even these bugs, there exists a necessity for the expanded development of antibacterial agents with features aimed at other potential antibacterial targets (6,7). Hence, the search for antibiotics with new scaffolds is of utmost importance at this juncture. While the search for new and novel class of molecules continues, mining of marine microbial products for such molecules is considered as an attractive alternative and have been receiving immense interest from the majority of drug discovery organizations all over the globe (8).

In our continuing efforts for finding a new and novel class of antibiotics and other drug candidates from marine microorganisms, we have discovered a novel and potent thiazolyl cyclic-peptide antibiotic molecule, PM181104 from the actinobacterium strain of the genera *Kokuria* isolated and characterised from the marine sponge *Spirastrella inconstans* var. *digitata* collected from the Palk Bay in Tamil Nadu, India (9). A recent report appeared in February, 2013 (10) claims to have isolated a molecule called kocurin from a marine derived strain of *Kocuria palustris* F-276,345. Kocurin was also reported to have been isolated from *Kokuria* sp. strain MI-67-EC3-038 (11). Interestingly the former report (10) claims that kocurin and the compound PM181104 are identical molecules and structure of kocurin (Fig. 1) is indeed more acceptable structure of PM181104. The compound PM181104 has been studied by us extensively and has been discovered to possess potent *in vitro* anti-Gram-positive activity and *in vivo*

efficacy in different murine models. In the current report details of the *in vitro* and *in vivo* studies have been presented.

Materials and Methods

General Experimental Procedures

All reagents and chemicals were obtained from Sigma and Merck unless otherwise specified. Linezolid was obtained from Glenmark Pharma Ltd. Amphotericin B, Fluconazole and vancomycin were purchased from Hi-Media. UV spectra were recorded on a JASCO spectrophotometer equipped with a peltier temperature control unit. The sample was dissolved in DMSO. All relevant animal procurement and study protocols were reviewed and requisite permissions obtained from the Institutional Animal Safety Committee, before initiating the *in vivo* efficacy studies.

In vitro antibacterial activity

Minimum Inhibitory Concentration (MIC) values for the test and standard antibiotics were determined in Mueller Hinton Broth by the broth macrodilution method according to NCCLS guidelines, as per document no. M7-A5 (12). The medium with a twofold serial dilution of compound in Mueller Hinton broth was inoculated with 10^5 colony forming units/mL of test culture and incubated at 37°C for 24 hrs. The MIC of the PM181104 was determined against 261 test organisms including clinical and standard strains of *S. aureus*, both MRSA and methicillin-sensitive *S. aureus* (MSSA), clinical and in-house strains of Enterococci, both VRE and Vancomycin sensitive enterococci (VSE), *S.epidermidis* strains, *Bacillus spp* and 17 gram negative strains. American type culture collection (ATCC) strains were also screened. Methanol was used as solvent for making stock solutions of test compound. Appropriate controls (positive, negative, solvent, and medium) were used in the assay, and the tubes were observed for growth of test culture in terms of turbidity visible to the naked eyes after 24 hrs of incubation. The MIC values of the compounds were recorded in µg/mL and expressed as MIC range, MIC₅₀ and MIC₉₀ for the test compound. MIC₉₀ and MIC₅₀ values were defined as the lowest concentration of the antibiotic at which 90% and 50% of the isolates were inhibited, respectively.

Linezolid and vancomycin were used as standard antibacterial agents.

In vitro antifungal activity

MIC values for the test and standard antifungals were determined by the NCCLS (CLSI) Macrobrotth dilution method for yeasts as per document no. M27-A2, (13) and for filamentous fungi as per document no. M38-A (14). The microbes were grown on Sabouraud agar slants at 37°C for 48 h and the inoculum was prepared in sterile saline. Optical density of the suspension was adjusted to 1.0 at 600 nm so as to obtain 10^8 colony forming units (cfu)/mL for *Candida* species. The final working inoculum was 0.5×10^4 cfu/mL. For *Aspergillus* species, the inoculum was prepared in sterile saline containing 0.01% Tween 80 for uniform distribution of the spores. The percent transmittance was adjusted between 80-82% at 530 nm so as to produce 10^6 spores/mL. The final working inoculum was 0.5×10^4 spores/mL. Test compound was suitably diluted in the concentration range of 0.5 – 256 µg/mL. The MIC assay tubes were incubated at 37°C for 48 hrs. Appropriate controls (positive, negative, solvent, and medium) were used in the assay, and the tubes were observed for growth of test culture in terms of turbidity visible to the naked eyes after 48 hrs of incubation. The MIC values of the compounds were recorded in µg/mL. Amphotericin B and Fluconazole were used as standard antifungal compounds.

In vivo activity of PM181104

Preparation of bacterial culture for infection

MRSA (strains E710 and ATCC 33591) and VRE (strain ATCC 47077) cultures were grown overnight in Brain heart infusion broth. Log phase cultures were prepared by sub-culturing the overnight growth into fresh broth and incubating for two hours. The log-phase cultures were centrifuged and the cell pellets were suspended in normal saline (0.85% sodium chloride solution). These culture suspensions were diluted further in normal saline to obtain the required colony forming units (cfu) of bacteria for infection.

In order to test the efficacy of PM181104 in animals, an injectable formulation comprising 6% (w/w) PEG 400 and 8% (w/w) Tween-80 was used. It was prepared by dissolving PM181104 in the excipients mixture and diluted using water for injection (WFI). PM18104 was dissolved in this mixture and then diluted using sterile water for injection.

Efficacy of PM181104 against MRSA in septicemia model

Balb/c mice (6-8 weeks of age, 19-22g) were randomly assigned into 7 different treatment groups (6 mice per group) and infected intra-peritoneally with 0.1mL of MRSA strain E710 culture containing 10^8 - 10^9 cfu of bacteria. PM181104 prepared in formulation was administered at 1.25, 2.5, 5 and 10mg/kg intra-venous (i.v.) dose immediately post infection to the assigned animals. One group received formulation excipients (vehicle control) by i.v. route and one group received 25mg/kg standard antibiotic linezolid (obtained from Glenmark Pharma Ltd.; Batch # K2005028) by intra-peritoneal (i.p.) route. The dosing volume was 10mL/kg body weight. One group of infected mice received no treatment (infection control). Mice were observed for mortality and morbidity up to 10 days.

Efficacy of PM181104 against VRE in septicemia model

Six-eight week old Balb/c mice were randomized into 6 groups (6 mice per group) and were infected intra-peritoneally with 2.87×10^9 cfu of VRE strain, *E. faecium* ATCC 47077 culture. PM181104 was administered at 2.5, 5 and 10mg/kg intra-venous (i.v.) dose immediately post infection to 3 different groups. One group received formulation excipients (vehicle control) by i.v. route and one group received 25mg/kg standard antibiotic linezolid by intra-peritoneal (i.p.) route. The dosing volume was 10mL/kg. One group of infected mice received no treatment (infection control). Mice were observed for mortality and morbidity up to 10 days.

***In vivo* time-kill curve of VRE**

The neutropenic mice were infected in the thigh with 0.1 mL of VRE strain ATCC 47077 culture, containing 4.16×10^6 cfu of bacteria. Three mice were sacrificed immediately post-infection and their thighs were removed and processed for cfu determination. Two hours after infection, 30 mice were treated with single i.v. dose of PM181104 (10 mg/kg) prepared in formulation PM-FO-52. PM-FO-52 represents 8% (w/w) PEG 400 and 8% (w/w) Tween-80 was prepared. PM181104 was dissolved in this mixture and then diluted using 0.9% Saline. Another group of 30 mice were treated with blank formulation excipients (vehicle control) i.v. and the third group of 30 mice received 25mg/kg standard antibiotic linezolid intraperitoneally. The dosing volume was 10mL/kg for all the groups. Six mice were sacrificed at the time of dosing and their thighs harvested for bacterial count determination. Six mice from each of the three treatment groups, (viz. PM181104, vehicle control and standard antibiotic) were

sacrificed at five sampling times over a time-interval starting from 2 hours post dosing up to 24 hours post dosing. The thighs were removed at each time point and processed for cfu determination.

Efficacy of PM181104 against MRSA in murine lung infection model

Female Balb/c mice, weighing between 18 and 21 g, were rendered neutropenic by treatment with 200 mg/kg of cyclophosphamide (Sigma, Cat # C0768) intraperitoneally at 4 and 2 days prior to infection. The mice were anesthetized with i.p. injection of a mixture of ketamine (50mg/kg) and xylazine (10mg/kg) and held in an upright position to be inoculated. Each animal was infected by placing 50 μ l of bacterial inoculum containing approximately 2.7×10^6 cfu of MRSA strain E710 onto the tip of the nares. The animals were allowed to inhale the inoculum as small droplets and were then placed back into their respective cages for recovery and observation. Dosing of PM181104 was done 24 and 36 hours post-infection. The dosing volume was 10mL/kg for all the groups and the group size for all experimental groups was 6 mice per group. PM181104 was administered at 2.5, 5 and 10mg/kg intravenous dose to the pre-assigned groups. Formulation excipients mix of the formulation PM-FO-52 was administered by the same route and similar dosing volume to the control group. 25mg/kg standard antibiotic linezolid by i.p. route and 110mg/kg standard antibiotic vancomycin by i.v. route was administered to the two positive control groups. Mice were euthanized 48 hours post-infection and their lungs collected for bacterial count determination.

Efficacy of PM181104 against VRE in murine kidney infection model

Efficacy of PM181104 was tested in λ -carrageenan –induced kidney infection model (15). Female Balb/c mice were pretreated with 0.2mL of 2% λ -carrageenan, injected intravenously seven days prior to bacterial challenge. VRE culture at a bacterial density of 8.29×10^9 cfu/mL was used to infect the mice. Mice were infected with 0.2mL of the culture suspension containing 1.66×10^8 cfu, intravenously. PM181104 was administered at 5, 10 and 15mg/kg i.v. dose, 4 hours post infection, and continued once daily for 3 consecutive days. Two control groups, one treated with formulation excipients (group size, n = 4) and one without any treatment, but only infection formed part of the study. Two standard antibiotics were selected as positive controls – linezolid (25mg/kg/day; i.p.) and vancomycin (150mg/kg/day, i.v.) The group size was 4 to 6 mice per group. Mice were euthanized and their kidneys harvested 3 days post-infection.

Results and Discussion

In vitro activity

The compound PM181104 exhibited potent growth inhibitory activities against Gram positive bacterial strains (Table 1). It inhibited the growth of *S. aureus* (both MRSA and MSSA) with minimum inhibitory concentration (MIC) in the range of 0.008-2.048 $\mu\text{g/mL}$ and MIC₉₀ value of 0.064 $\mu\text{g/mL}$. The compounds also acted on *S.aureus*- cfr and linezolid resistant strains of *S.aureus* LZD^R. It was effective against *S. epidermidis* with the MIC range of 0.008-1.024 $\mu\text{g/mL}$ and MIC₉₀ value of 0.128 $\mu\text{g/mL}$. PM181104 showed better potencies against VRE and VSE strains exhibiting a MIC range of 0.004-1.024 $\mu\text{g/mL}$ and MIC₉₀ value of 0.016 $\mu\text{g/mL}$. It also inhibited doxycycline-resistant strains of *E. faecium* and *E. faecalis* as well as linezolid and gentamicin resistant strains of *E.faecalis*. Clinical strains of *Enterococci* were inhibited by the compound in the MIC range of 0.004-0.128 $\mu\text{g/mL}$ with a MIC₉₀ value of 0.064 $\mu\text{g/mL}$. Bacillus strains were also strongly inhibited by the compound with MIC range of 0.004-0.016 $\mu\text{g/mL}$. Apart from MIC estimation, the compound was also tested by agar well diffusion assays against 17 Gram negative strains (*E.coli*, *Klebsiella* spp., *Salmonella* spp., Shigella, *Acinetobacter baumannii*, *Pseudomonas* spp.) and four strains of fungi [*Candida albicans* (I.V.), *C. albicans* ATCC 14503, *Aspergillus fumigatus* (HMR) and *A. fumigatus* ATCC 16424] at a concentration up to 256 $\mu\text{g/mL}$. There was no zone of inhibition around any agar well for any of these test culture. Thus, we confirmed that none of the Gram negative test organisms and fungal strains was inhibited by the compound PM181104. Therefore compound PM181104 showed good *in vitro* activity against different strains of Gram positive test organisms, including resistance strains. Later it was considered for its *in vivo* efficacy.

In vivo efficacy

Efficacy of PM181104 against MRSA in septicemia model

Morbidity and mortality was observed in the vehicle control and infection control groups within three days of infection. The vehicle offered no protection from infection as all the infected animals died by day 3. At 1.25mg/kg dose, three mice survived up to day 10. All mice that received 2.5 and 5mg/kg dose of PM181104 survived up to day 10 post-infection. Fig. 2 shows Kaplan-Meier survival plot of mice up to 10 days. PD₁₀₀ for PM181104 was 2.5mg/kg.

Efficacy of PM181104 against VRE in septicemia model

100% mortality was observed in the vehicle control and infection control groups within 2 days of infection. At 2.5mg/kg dose, three mice survived up to day 10 and at 5mg/kg dose, 4mice survived up to day 10. All mice that received 10mg/kg dose of PM181104 survived up to day 10 post-infection. Fig. 3 shows Kaplan-Meier survival plot of mice up to 10 days. PD₁₀₀ for PM181104 was 10mg/kg.

***In vivo* time-kill curve of VRE**

The culture showed exponential growth phase up to 9 hours in the control mice. In PM181104-treated mice, the bacterial growth up to 2 hours was similar to that in the control group. However, between 4 to 9 hours, there was a insignificant reduction in the bacterial growth in PM181104 treated mice as compared to the control with a difference of 0.48 and 0.42 log cfu/g at 4 and 9 hours respectively which was not comparable to that of linezolid. However at 9 hours the mean bacterial titer in PM181104 treated mice was at par to that of linezolid standard. Fig. 5 shows the mean log cfu/g for the three treatment groups at different time-points up to 24 hours.

Efficacy of PM181104 against MRSA in murine lung infection model

The bacterial titer in the lungs of the treated mice was determined 48 hours post-infection. PM181104 showed a decrease of 0.74 log cfu/g at 2.5mg/kg dose, 0.54 log cfu/g at 5mg/kg dose and 0.68 log cfu/g at 10 mg/kg dose as compared to the control. Standard antibiotics linezolid and vancomycin showed bacterial count reduction of 0.90 log cfu/g and 1.56 log cfu/g, respectively as compared to the control. There was no dose response for PM181104 against MRSA in the murine lung infection model which indicates the poor availability of the antibiotic in lung tissues when injected through i.v. route.

Efficacy of PM181104 against VRE in murine kidney infection model

Untreated control mice showed no signs of systemic infection and the infection was localized in the kidneys. PM181104 showed a decrease of 0.28 log cfu/g at 5mg/kg dose, 0.69 log cfu/g at 10mg/kg dose and 0.73 log cfu/g at 15 mg/kg dose as compared to the infection control. PM181104 showed a dose-dependent reduction in bacterial titer in the kidney infection. Standard antibiotics linezolid and vancomycin showed bacterial count reduction of 0.53 log cfu/g and 1.31 log cfu/g, respectively as compared to the control. Fig. 8 shows the mean bacterial titer \pm SD in the different treatment groups.

Discussions

There are several molecules reported in this class of molecules (10, 16). Most of them represented very potent *in vitro* activity against Gram positive strains especially MRSA. However the development of this class of compounds as clinical agents has been hindered due to low aqueous solubility and unfavorable pharmacokinetics. For PM181104 after several attempts a suitable formulation could be developed by us, which supported animal studies of PM181104.

In vivo efficacy of PM181104 was tested against MRSA in general purpose models as well as tissue or organ – specific infection models. In the general purpose efficacy testing model of systemic disseminated septicemia, the mice were challenged with a lethal dose of MRSA strain E710 intraperitoneally and subsequently treated intravenously with different doses of PM181104. The compound showed 100% protection at a dose of 5mg/kg.

Anti-infective activity of PM181104 for organ or tissue-specific infection was evaluated in two mouse models – skin abscess model (data not presented) and lung infection model. The lung bacterial titer at 48 hours post-infection in PM181104 treated mice showed a bacteriostatic effect with 2 log difference in the bacterial count as compared to the control. In all the above described murine models, in some of the models efficacy of PM181104 was comparable to standard antibiotic Linezolid administered intraperitoneally at 25mg/kg dose.

In vivo antibacterial activity of PM181104 against VRE was tested in mice in λ -carrageenan -induced kidney infection model. The bacterial count in the kidneys 72 hours post-infection in the mice treated with 5mg/kg dose of PM181104 showed a reduction of 1 log cfu as compared to the control. The efficacy of PM181104 in treating kidney-infection was comparable to standard antibiotics – Linezolid at 25mg/kg dose and Vancomycin at 150mg/kg dose.

The *in vivo* activity of PM181104 has implications as an anti-infective therapeutic agent. The potent *in vitro* activity and successful *in vivo* results emphasize that the PM181104 class of compounds provides a great potential for the development of vitally needed antibiotics. However, further studies of pharmacokinetic parameters, toxicity and development of a suitable formulation for human administration will decide the path of this compound.

Our continued efforts to discover novel antibiotic molecules from marine microbial sources have led to the discovery of PM181104, a potent thiazolyl cyclic-peptide molecule. In *in vitro* studies the antibiotic PM181104 demonstrated potent anti-gram positive antibacterial activity, especially against drug resistant bacteria such as MRSA and VREs. The *in vitro* activity got translated well into *in vivo* models. The molecule was able to protect the mice from systemic infection as well as organ specific infections. Antibiotic PM181104 or its variants reported elsewhere represent a promising scaffold for the treatment of gram positive bacterial infections especially by *S. aureus* as well as enterococcal infections caused by drug-resistant strains.

Acknowledgments

We would like to thank Dr. Somesh Sharma, and Dr. Arun Balakrishnan for their continuous help and encouragement during the course of this work. Also we would like to thank to Council of Scientific and Industrial Research (CSIR)-National Institute of Goa-India the collaborative partner in this work for providing the marine samples.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. **Menichetti, F.** 2005. Current and emerging serious Gram-positive infections. *Clin. Microbiol. Infect.* **11**(Suppl. 3):22–28.
2. **Aeschlimann, J. R., and M. J. Rybak,** 1998. Pharmacodynamic analysis of the activity of quinupristin-dalfopristin against vancomycin-resistant *Enterococcus faecium* with differing MBCs via time-kill-curve and postantibiotic effect methods. *Antimicrob. Agents Chemother.* **42**:2188–2192.
3. **Tenover, F. C., L. M. Weigel, P. C. Appelbaum, L. K. McDougal, J. Chaitram, S. McAllister, N. Clark, G. Killgore, C. M. O’Hara, L. Jevitt, J. B. Patel, and B. Bozdogan.** 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. *Antimicrob. Agents Chemother.* **48**:275–280.
4. **Infectious Diseases Society of America.** 2010. The 10 X ’20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clin. Infect. Dis.* **15**:1081–1083.

5. **Boucher, H. W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B, and Bartlett, J.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48**:1–12.
6. **Mutnick, A.H., Enne, V., and Jones, R.N.** 2003. Linezolid resistance since 2001: SENTRY Antimicrobial Surveillance Program. *Annals of Pharmacotherapy.* **37**: 769–74.
7. **Skiest, D. J.** 2006. Treatment failure resulting from resistance of *Staphylococcus aureus* to daptomycin. *J. Clin. Microbiol.* **44**: 655–656.
8. **Mayer, A.M.S., Glaser, K.B., Cuevas, C., Jacobs, R.S., Kem, W., Little, R.D., McIntosh, J.M., Newman, D.J., Potts, B.C., and Shuster, D.E.** 2010. The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol. Sci.* **31**: 255-265.
9. **Mahajan, G.B., George, S.D., Ranadive, P.V., Mishra, P.D.S., Eyyammadichiyil, S.S., Panshikar, R.M., Sawant, S.N., Krishna, S., Sivakumar, M., Pari, K., Prabha, D., D'Souza, L., Naik, C.G., Patel, Z.E., Sivakumar, M., Thomas, B., and Vishwakarma, R.** 2007. PM181104 and Related Antibacterial Compounds, Production, Pharmaceutical Compositions, and Therapeutic Use. Int PCT, App. WO/2007/119201 A3.
10. **Martín, J., da S. Sousa, T., Crespo, G., Palomo, S., González, I., Tormo, J.R., de la Cruz, M., Anderson, M., Hill, R.T., Vicente, F., Genilloud, O., and Reyes, F.** 2013. Kocurin, the True Structure of PM181104, an Anti-Methicillin-Resistant *Staphylococcus aureus* (MRSA) Thiazolyl Peptide from the Marine-Derived Bacterium *Kocuria palustris*. *Mar. Drugs.* **11**: 387-398.
11. **Cañedo Hernández, L.M., Romero Millán, F., Fernández Medarde, A., Fernández Chimeno, R.I., Hidalgo Villar, J.C.** 2012. Peptides as bioactive compounds. Int PCT, App. WO/2012/062906.
12. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5, 5th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
13. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard, 2nd ed. NCCLS document M27-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.
14. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard. NCCLS document M38-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
15. **Alder, J., T. Li, D. Yu, L. Morton, J. Silverman, X. X. Zhang, I. Critchley, and G. Thorne.** 2003. Analysis of daptomycin efficacy and breakpoint standards in a murine model of *Enterococcus faecalis* and *Enterococcus faecium* renal infection. *Antimicrob. Agents Chemother.* **47**:3561–3566.
16. **Zhang, C., Herath, K., Jayasuriya, H., Ondeyka, J.G., Zink, D.L., Occi, J., Birdsall, G., Venugopal, J., Ushio, M., Burgess, B., Masurekar, P., Barrett, J.F., and Singh, S.B.** 2009. Thiazomycins, Thiazolyl Peptide Antibiotics from *Amycolatopsis fastidiosa*. *J. Nat. Prod.* **72(5)**: 841-847.

Figure legends:

FIG 1 Structure of PM181104 (=Kocurin) (10)

FIG 2. *In vivo* antibacterial activity of PM181104 against *S.aureus*-E710 (MRSA) in murine septicemia model when administered i.v. at 1.25mg/kg, 2.5mg/kg and 5 mg/kg compared to the vehicle control, untreated control and standard antibiotic – linezolid control given i.p at 25mg/kg dose. Survival was monitored for 10 days. [Linezolid in the figure has to be labeled at 25mg/kg, Rutuja]

FIG 3 *In vivo* antibacterial activity of PM181104 against *E. faecium* ATCC 47077 (VRE) in murine septicemia model when administered i.v. at 2.5mg/kg, 5mg/kg and 10 mg/kg compared to the vehicle control untreated control and standard antibiotic – linezolid control given i.p at 25mg/kg dose. Survival was monitored for 10 days.[The X-axis ticks are not aligned with the labeled times. State doses administered in mg/kg in the box-Rutuja]

FIG 4 *In vivo* time-kill curve of *E. faecium* ATCC 47077 (VRE) in neutropenic thigh model with i.v. administration of 10mg/kg dose of PM181104 compared to the vehicle control and standard antibiotic – linezolid control given i.p at 25mg/kg dose.[The X-axis ticks are not aligned with the labeled times. State doses administered in mg/kg in the box, Rutuja]

FIG 5 *In vivo* antibacterial activity of PM181104 against *E. faecium* ATCC 47077 in murine kidney infection model when intravenously administered once daily at 5mg/kg, 10mg/kg and 15 mg/kg for 3 days, compared to the control and two standard antibiotics, viz. linezolid given i.p at 25mg/kg dose and vancomycin given i.v. at 150mg/kg dose.

Table legends

TABLE 1 *In vitro* antibacterial activity of antibiotic PM181104

Test culture (n)	MIC range ¹ (µg/mL)	MIC ₅₀ ² (µg/mL)	MIC ₉₀ ³ (µg/mL)
<i>S. aureus</i> MRSA (67)	0.008-2.048	0.032	0.064
<i>S. aureus</i> MSSA (48)	0.008-2.048	0.032	0.064
<i>S. aureus</i> MRSA, inducible ERY ^R (1)	1.024	-	-
<i>S.aureus</i> cfr (1)	1.024	-	-
<i>S.aureus</i> LZD ^R (6)	0.250-0.500	-	-
<i>S.epidermidis</i> (23)	0.008-1.024	0.064	0.128
<i>E.faecium</i> VSE (12)	0.008-0.032	0.008	0.016
<i>E.faecalis</i> VRE (12)	0.064-1.024	0.064	0.128
<i>E.faecalis</i> VSE (30)	0.002-16	0.008	0.016
<i>E.faecium</i> VRE (5)	0.008-0.008	-	-
<i>E.faecium</i> D ^R (3)	0.128-0.256	-	-
<i>E.faecalis</i> LZD ^R (5)	0.128-0.256	-	-
<i>E.faecalis</i> GM ^R (6)	0.128-0.512	-	-
<i>E.faecalis</i> D ^R (3)	0.256-0.512	-	-
Clinical Enterococci spp.VRE (16)	0.004-0.128	0.008	0.064
Bacillus spp.(6)	0.004-0.016	-	-

MRSA: methicillin-resistant *Staphylococcus aureus*; MSSA: methicillin-sensitive *Staphylococcus aureus*; ERY^R: erythromycin-resistant; LZD^R: linezolid-resistant; VRE: vancomycin-resistant enterococci; VSE: vancomycin-susceptible enterococci; D^R: doxycycline-resistant; GM^R: gentamicin-resistant; cfr: gene which mediates resistance phenotype includes oxazolidinones, pleuromutilins—phenicols, lincosamides, and streptogramin A antibiotics etc; n: number of test strains. ¹:Range of MIC values; ²: Concentration required to inhibit 50% of the test strains; ³: Concentration required to inhibit 90% of the test strains

TABLE 2 Comparative *in vitro* antibacterial activity of PM181104 and standard antibiotic.

Test organisms (n)	MIC ($\mu\text{g/mL}$)	
	PM181104	Linezolid
<i>S. aureus</i> ATCC 3066, MRSA*, ERY ^R	0.032	0.512
<i>S. aureus</i> E710, MRSA, ERY ^R	0.016	2.560
<i>S. aureus</i> ATCC 33591, MRSA*	0.032	2.560
<i>E. faecium</i> (02 D3 IP1), VRE ^S , Teicho ^R	0.008	2.560
<i>E. faecium</i> (R-2), VRE ^S (VanA)	0.008	2.560
<i>E. faecalis</i> ATCC 29212, VSE ^{SS}	0.016	2.560
<i>E. faecium</i> ATCC 19579	0.032	2.560
<i>E. faecalis</i> ATCC 51299, VRE ^S , GM ^R , STR ^R (VanB)	0.016	2.560
<i>E. faecalis</i> ATCC 47077	0.032	1.280
<i>E. faecalis</i> LZD ^R (5)	0.128-0.256	8-32
<i>E. faecalis</i> GM ^R (6)**	0.128-0.512	0.5-4
<i>E. faecalis</i> D ^R (3)***	0.256-0.512	1-2

MRSA: methicillin-resistant *Staphylococcus aureus*; ERY^R: erythromycin-resistant; LZD^R: linezolid-resistant; VRE: vancomycin-resistant enterococci; VSE: vancomycin-susceptible enterococci; D^R: doxycycline-resistant; GM^R: gentamicin-resistant; STR^R: streptomycin-resistant; Teicho^R: teichoplanin resistant; n: number of test strains; * MIC of oxacillin for these strains is >64 $\mu\text{g/mL}$; ** MIC range of gentamicin for these strains is >32 $\mu\text{g/mL}$; ^S MIC of vancomycin for these strains is >64 $\mu\text{g/mL}$; ^{SS} MIC of vancomycin for these strains is 0.5-2 $\mu\text{g/mL}$; *** MIC range of oxacillin for these strains is 16-32 $\mu\text{g/mL}$.

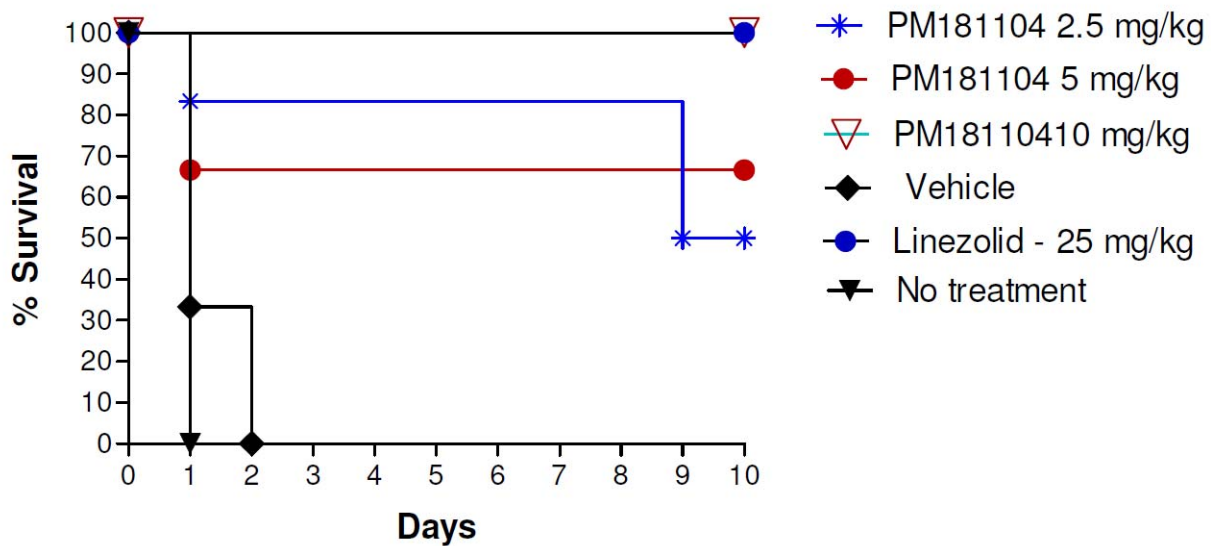


Fig 3 *In vivo* antibacterial activity of PM181104 against VRE in murine septicemia model when administered i.v. at 1.25mg/kg, 5mg/kg and 10mg/kg compared to the vehicle control, untreated control and standard antibiotic – linezolid control given i.p. at 25mg/kg dose. Survival was monitored for 10 days.

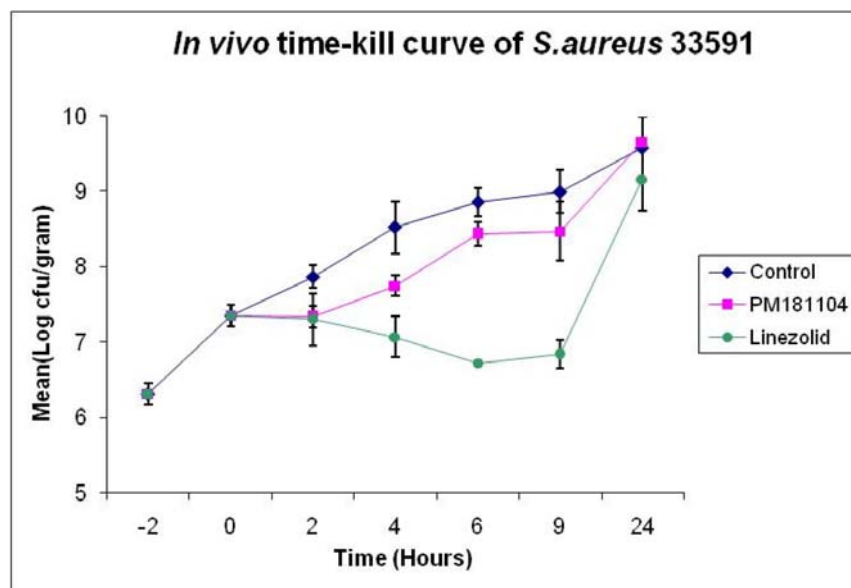


FIG 4 shows in vivo time-kill curve of MRSA in neutropenic thigh model with i.v. administration of 5mg/kg dose of PM181104 compared to the vehicle control and standard antibiotic – linezolid control given i.p. at 25mg/kg dose.

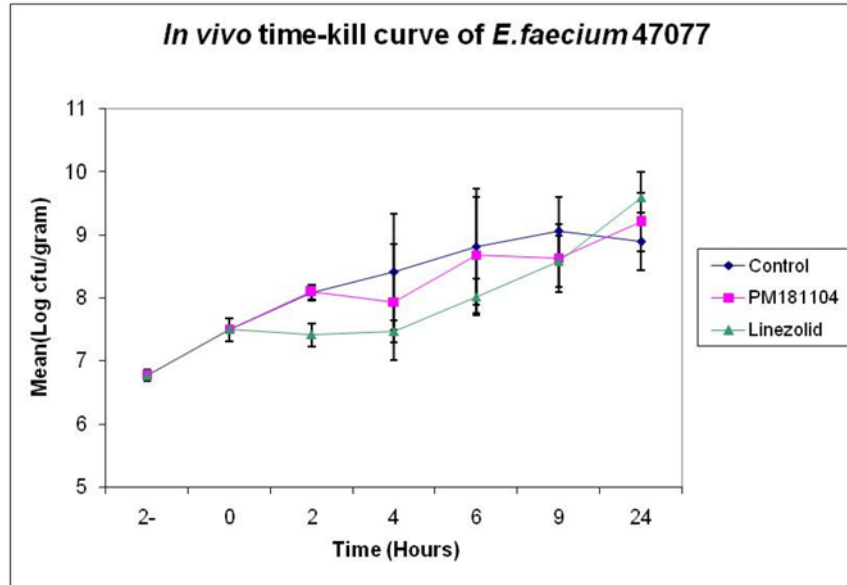


FIG 5 shows in vivo time-kill curve of VRE in neutropenic thigh model with i.v. administration of 10mg/kg dose of PM181104 compared to the vehicle control and standard antibiotic – linezolid control given i.p. at 25mg/kg dose.

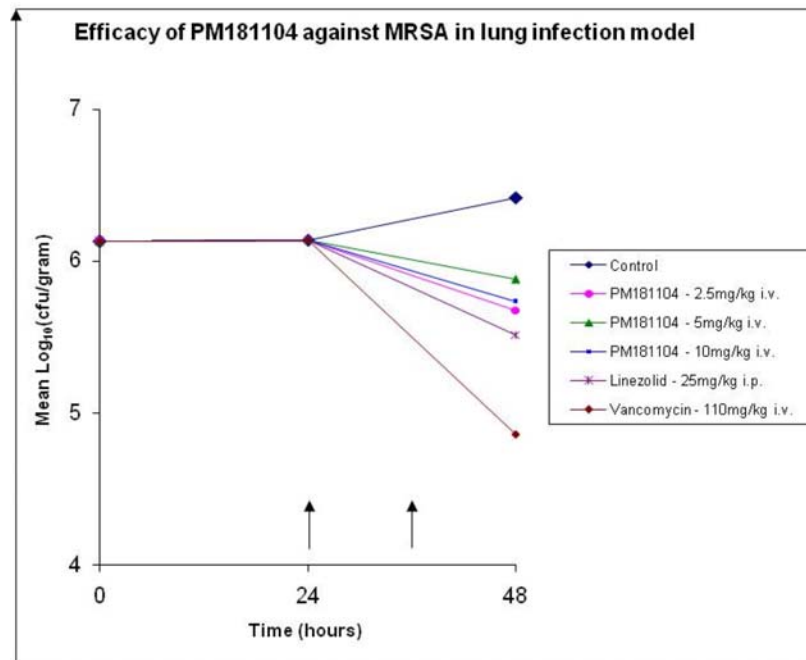


FIG 6 shows in vivo antibacterial activity of PM181104 against MRSA in murine lung infection model when administered i.v. at 2.5mg/kg, 5mg/kg and 10 mg/kg compared to the vehicle control and two standard antibiotics – linezolid given i.p. at 25mg/kg dose and vancomycin given at 110mg/kg iv dose. Arrows indicate the dosing times.

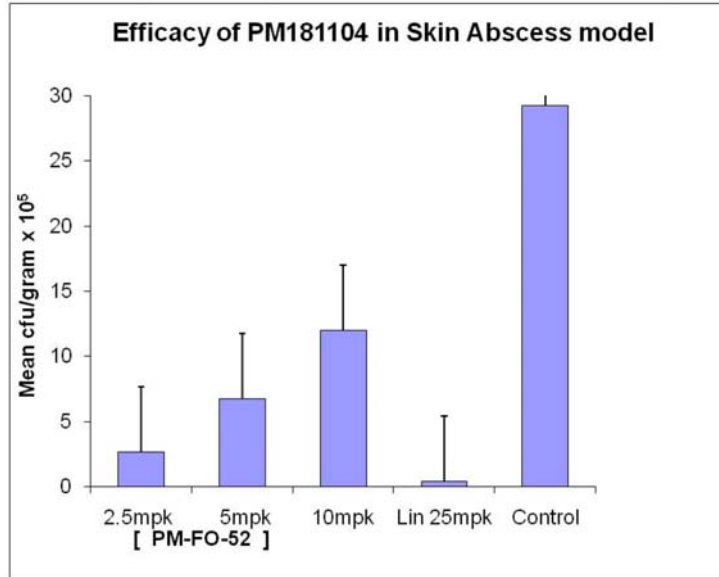


FIG 7 shows *in vivo* antibacterial activity of PM181104 against MRSA in murine skin abscess model when administered i.v. at 2.5mg/kg, 5mg/kg and 10 mg/kg compared to the control and a standard antibiotic – linezolid given i.p. at 25mg/kg dose.

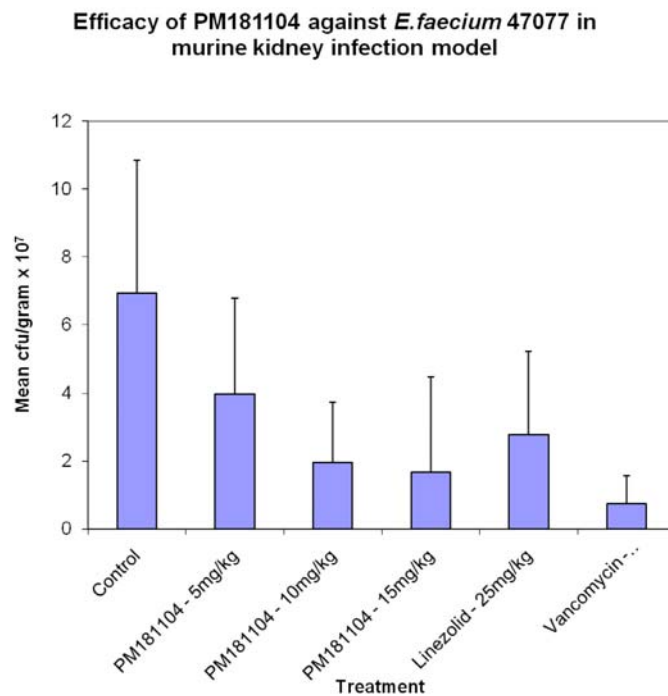


FIG 8 shows *in vivo* antibacterial activity of PM181104 against MRSA in murine kidney infection model when intravenously administered once daily at 5mg/kg, 10mg/kg and 15 mg/kg for 3 days, compared to the control and two standard antibiotics, linezolid given i.p. at 25mg/kg dose and vancomycin given at 150mg/kg dose.

TABLE 3 *In vitro* antifungal activity of antibiotic PM181104 and Amphotericin B

Test organism	MIC ($\mu\text{g/mL}$) of compounds	
	PM181104	Amphotericin B
<i>C. albicans</i> (I.V.)	>256	0.256
<i>C. albicans</i> ATCC 14503	>256	0.256
<i>A. fumigatus</i> (HMR)	>256	0.256
<i>A. fumigatus</i> ATCC 16424	>256	0.313