J Biosci (2020)45:147
DOI: 10.1007/s12038-020-00113-7

Pharmacological characterization of a structurally new class of antibacterial compound, triphenyl-phosphonium conjugated diarylheptanoid: Antibacterial activity and molecular mechanism

Shweta Kumari[1,†], Sundarraj Jayakumar[1,†], Subhash C Bihani[1,†], Neena Shetake[1,4], Raji Naidu[2], Vijay K Kutala[3], Haladhar D Sarma[1], Gagan D Gupta[1,4], Santosh K Sandur[1,4]* and Vinay Kumar[4]*

[1]*Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400 085, India*

[2]*Medical Division, Bhabha Atomic Research Centre, Mumbai 400 085, India*

[3]*Department of Clinical Pharmacology and Therapeutics, Nizam's Institute of Medical Sciences, Hyderabad 500 082, India*

[4]*Training School Complex, Homi Bhabha National Institute, Anushaktinagar, Mumbai 400 094, India*

[†]The authors have contributed equally to this work.

**Corresponding authors (Emails, sskumar@barc.gov.in; vkbhatia@gmail.com)*

MS received 25 May 2020; accepted 9 October 2020

Many pathogenic species of bacteria are showing increasing drug resistance against clinically used antibiotics. Molecules structurally distant from known antibiotics and possessing membrane targeting bactericidal activities are more likely to display activity against drug-resistant pathogens. Mitocurcumin (MitoC) is one of such compounds, synthesized by triphenyl-phosphonium conjugation with curcumin, and has been shown recently from our laboratory to have broad-spectrum bactericidal activity (Kumari *et al.* 2019 *Free Radic. Biol. Med.* **143** 140–145). Here, we further demonstrate the antibacterial properties of MitoC against resistant strains and also its mechanism of action. It displays efficient bactericidal activity against multidrug-resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* (MIC values in the 1.5–12.5 μM range), and coagulase-negative *Staphylococci* do not show resistance development against MitoC. Liposome based studies and MIC values against TolC deletion mutant (Δ*tolC*; outer membrane protein) of *E. coli* suggest extensive membrane damage to be the primary mechanism of bactericidal activity. MitoC did not exhibit toxicity in BALB/c mice with an oral administration of 250 mg/kg body weight and was found to be totally safe without any significant effect on haematological, biochemical parameters and inflammatory responses. Its rapid bactericidal action as assessed by *in vitro* time-kill assay against *B. subtilis*, compared to ciprofloxacin, and long half-life in rodent serum, suggest that MitoC could be an excellent lead-molecule against drug-resistant pathogens. The highlights of the study are that mitocurcumin belongs to a structurally new class of bactericidal compounds. It displays activity against MDR strains of pathogenic bacteria and challenging MRSA. Liposome-based studies confirm the membrane damaging property of the molecule. Mitocurcumin does not show resistance development even after 27 bacterial generations.

Keywords. Bactericidal; drug-resistance bacteria; liposomal models; mitocurcumin; new structural class antibiotics; pharmacological properties; time-kill assay

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s12038-020-00113-7) contains supplementary material, which is available to authorized users.

http://www.ias.ac.in/jbiosci
Published online: 24 November 2020

1. Introduction

Most of the 'last resort' and new antibiotics belong to the structural class of one of the currently used antibiotics. Due to the similarity of structures, these antibiotics are vulnerable to rapid resistance development. Therefore, there is an urgent need for the discovery of antibacterial compounds belonging to a new structural class, given the growing antimicrobial resistance and our over-dependence on antimicrobial molecules. Natural products are a rich source of antimicrobial compounds. Curcumin has been shown to have antimicrobial, antioxidant, antitumor and anti-inflammatory activities but its clinical application is limited due to poor pharmacokinetics and bioavailability. Recently, we reported broad-spectrum bactericidal activity of mitocurcumin (MitoC), which is a novel compound synthesized by triphenyl-phosphonium (TPP) conjugation with curcumin. Its bactericidal action was adjudged as due to the rapid disruption of bacterial membrane potential (Kumari *et al.* 2019). It was also earlier shown to have anti-cancer properties in human lung cancer cells (Jayakumar *et al.* 2017).

There are several mechanisms by which bactericidal drugs kill bacteria (Li *et al.* 2017). Resistance development towards molecules targeting bacterial cell membrane is expected to be slower as compared to conventional antibiotics (Kohanski *et al.* 2010). A variety of membrane-damaging mechanisms have been reported for different antibacterial agents (Kohanski *et al.* 2010). Use of membrane (liposomal) models of eukaryotic and prokaryotic cell membranes have been advocated for better understanding of the effect of membrane-acting antibiotics and for 'rational' drug-design (Pinheiro *et al.* 2019).

Microbial exposures result in global changes in the immune system response including induction of both the pro- and anti-inflammatory cytokines (Frieling *et al.* 1997). However, induced cytokine storm associated with severe inflammation increases the risk of immunopathologic injury, multi-organ dysfunction, etc. (Huggins *et al.* 2019). Antimicrobials also commonly affect the expression of cytokines on interaction with host and release of pro- or anti-inflammatory responses have been recorded for several antibiotics, including ceftazidime, amoxicillin (Alkharfy *et al.* 2000; Melhus 2001).

Dose and drug selection may mostly be based on a static *in vitro* parameter, minimum inhibitory concentration (MIC), and on the serum concentration of the drug as a pharmacokinetic parameter (Mueller *et al.* 2004). The MIC does not, however, provide information on the rate of bactericidal activity, and many different combinations of growth and kill rates can result in the same MIC. Further, serum concentrations do not reflect the unbound concentrations of the drug that is required at the therapeutic target. The recent *in vitro* approaches and modelling based on time-kill curves (microbial killing and growth based on both time and antibiotic concentration) are considered better representatives of drug pharmacokinetic and pharmacodynamic properties (Nielsen *et al.* 2007).

Here, we report efficient bactericidal activity of MitoC against multidrug-resistant bacteria and it does not show resistance development in *in vitro* assay. The wild-type and TolC deletion mutant of *E. coli* display the same sensitivity against the molecule. We have confirmed its membrane damage properties using liposome assays and find good consistency of MitoC activity towards lipid models and different cell types. Further, time-kill kinetics show rapid MitoC mediated killing at 4X MIC. A single oral safe-dose of the molecule does not significantly alter biochemistry, hematological and cytokine profiles in mice. It also displays good stability in rodent serum, most of it being in the unbound state.

2. Materials and methods

2.1 *Time-kill kinetics assay*

The *Bacillus subtilis* inoculum was prepared freshly by growing overnight from a single colony in Mueller–Hinton broth (MHB). Time-kill kinetics were performed in 96-well plate format with ciprofloxacin as reference antibiotic, in triplicate. An overnight culture of *B. subtilis* was diluted in MHB, such that the cell concentration was approximately 5×10^5 cells/ ml. Bacterial cells were then challenged with antibiotic and MitoC and were incubated at 37°C, and the viable count was performed at 0, 5, 10, 15, 30, 60 (min), 2, 4, 6 and 24 h. At specified intervals, 10 µl aliquots were removed, followed by serial microdilution in sterile MHB. A control was kept without the MitoC or reference antibiotic. The serially diluted cell suspension was plated on Mueller–Hinton Agar (MHA) plate and incubated at 37°C for 24 h. Bacterial colonies were counted and a graph of $\log_{10}$ (CFU/ml) *vs* time was prepared using Origin software.

2.2 *Serial passage resistance selection studies*

In-vitro resistance development assay was performed by sequential culturing of *Staphylococcus carnosus* in

presence of different concentrations of MitoC including sub-MIC level. Ciprofloxacin was included as a control. On day 1, MHB containing mitocurcumin or ciprofloxacin at 0.125, 0.25, 0.5, 1, 2 and 4 times the MIC was inoculated with 5×10^5 cfu of *S. carnosus*. Cultures were incubated overnight at 37°C with shaking. At 20-24 h intervals, the cultures were checked for growth. Cultures from the second-highest concentrations that allowed growth similar to non-treated cells ($OD_{600} \geq 2$) were diluted appropriately and inoculated into fresh media containing mitocurcumin/ciprofloxacin at 0.125, 0.25, 0.5, 1, 2 and 4 times the MIC. This serial passaging was repeated daily for 22 days in the case of ciprofloxacin while it was continued up to 27 days for mitocurcumin. After the experiment, cells showing resistance were passaged for 10 generations through antibiotic-free media and MIC was determined. Experiments were performed with biological replicates.

2.3 *Determination of MIC against TolC deletion mutant*

To assess the effect of the TolC efflux pump, MIC of MitoC was measured for *E. coli* TolC deletion mutant (Δ*tolC*) and compared with wild-type *E. coli*. Broth micro-dilution method as recommended by the Clinical and Laboratory Standard Institute (National Committee for Clinical Laboratory Standards, NCCLS) was used to determine MICs. In brief, MitoC was serially diluted in 50 µl of MHB in a 96-well microtiter plate. Freshly prepared inoculum of *E. coli* was added to each well of the 96-well microtiter plate to get a final concentration of 5×10^5 cells and was incubated for 18–20 h at 37°C at 300 rpm in a multi-mode plate reader (Clariostar, BMG Labtech, Germany). The bacterial growth was monitored by measuring OD_{600}. MICs were determined as the lowest concentrations of MitoC resulting in no increase in OD_{600} values in a multi-mode plate reader. Experiments were performed with biological replicates.

2.4 *Antibacterial activity of mitocurcumin against multi-drug resistant human pathogens*

Assay of antibacterial activity of MitoC against *S. aureus* clinical strains (MRSA and MDR) and *S. pneumoniae* (two clinical strains) was outsourced to a specialized infectious disease testing laboratory in Mumbai, India. MIC values for MitoC against these pathogens were determined in three independent assays using the NCCLS-recommended broth microdilution method.

2.5 *Liposome preparation*

For the present study carboxyfluorescein (CFL) encapsulated liposomes were used as model membranes for eukaryotic and Gram-negative cells. These were prepared by thin-film hydration method (Zhang 2017) using 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG) in varying ratios: (1) DSPC: DMPG (8:2; w/w) (eukaryotic liposomes) and (2) DSPE: DMPG (8:2; w/w) (Gram-negative liposomes) (Epand *et al*. 2007). We also attempted for preparing CFL encapsulated liposome as a mimic for Gram-positive cells using DSPE, DMPG and cardiolipin in stoichiometry (15: 80: 5).

Liposomes were prepared by dissolving appropriate amounts of lipids in chloroform/methanol (2:1, v/v), followed by solvent evaporation under a stream of nitrogen to deposit the lipid as a film on the walls of the test tube. Final traces of solvent were removed by vacuum drying the liposome preparations for 6 h. For making carboxyfluorescein-encapsulated liposomes, a thin film of phospholipids was hydrated in 1X PBS containing CFL fluorescent dye (100 mM) using glass beads and manual shaking with intermittent warming in a water bath at 45°C. These were sonicated using a titanium probe for 30 cycles (1 cycle: 45 s heat treatment at 52°C in a water bath followed by 15 s sonication) to obtain unilamellar liposomes (ULVs). The ULVs were then centrifuged at 2000 rpm for 5 min at 4°C to remove titanium particles. To obtain the uniform size distribution of the liposomes, the ULVs were passed through polycarbonate membranes of decreasing pore sizes (viz., 800 nm, 400 nm and 200 nm) using a membrane extrusion instrument (LiposoFast, Avestin Inc, France). The resulting uniform size ULVs were then passed through Sephadex G-50 column, followed by dialysis against normal saline (0.9% NaCl) for 2–4 h using 12 to 14 kDa MWCO dialysis membrane to remove the free CFL dye. The liposomes thus formed were stored at 4°C till further use. The size of the liposomes was determined by dynamic light scattering (DLS) after appropriate dilution in the PBS buffer.

2.6 *Liposomal disruption assay*

Membrane leakage was assessed by the de-quenching of CFL fluorescence (which is self-quenched at the high dye concentrations inside liposomes) after release from liposomes. The CFL-encapsulated liposomes were diluted in PBS (1:10) and 100 μl aliquots were incubated with different concentrations of MitoC (0.1–20 μM) at 37°C for 10 min and 2 hr. Percentage release of CFL was measured by further diluting the reaction mixture with PBS (1:100) and measuring the fluorescence in a plate reader (Tecan infinite Pro M200, Switzerland) at the excitation wavelength of 490 nm and emission at 520 nm. For 100% release of CFL, 10 μl aliquot of diluted liposomes was lysed with 10 μl of Triton X-100 (10%) or chloroform, followed by measuring the fluorescence in a plate reader. Disruption efficacy of MitoC against different liposome models was estimated by formula:

$$\text{Disruption efficacy} = (FL_N - FL_U) \times 100/(FL_L - FL_U) \quad (1)$$

where FL_N is fluorescence intensity observed on MitoC treatment, FL_U is fluorescence for untreated liposomes, and FL_L is fluorescence for completely lysed liposomes treated with triton X-100 or chloroform.

2.7 *Evaluation of acute toxicity of MitoC in mice*

Acute oral toxicity of MitoC in BALB/c mice was estimated by administering different doses ranging from 25 mg/kg to 500 mg/kg body weight. Mice were observed for any symptoms of toxicity, morbidity or mortality for 10 days. Similarly, the acute toxicity of MitoC in mice through the intraperitoneal route was also estimated by administering MitoC with a dose ranging from 2 mg/kg to 20 mg/kg body weight. Control mice were administered with DMSO as vehicle control.

2.8 *Evaluation of haematological and biochemical parameters, and serum cytokines*

Mice were orally administered with the safe-dose of MitoC (250 mg/kg body weight). Blood was collected from the mice for haematological and biochemical assessment 48 h after MitoC administration. Heparinized blood samples were evaluated for various blood parameters on the hematology analyzer (Orphee Mythic-200). However, the differential counts were done manually on blood smears stained by field stain. The serum was separated for biochemical analysis. Serum biochemical analysis was done with the help of a fully automatic clinical chemistry analyser, Rx Daytona + (Randox Laboratories Ltd., UK).

For evaluation of cytokine profile, blood was collected 24 h after MitoC administration and serum was separated from blood samples. Pro-inflammatory cytokines (IL-6, TNF-α and IFN-γ) were measured by cytokine ELISA kits by following the manufacturer's protocol (Becton Dickinson, USA). Cytokines were measured individually from three mice in each group and the average of these three mice have been plotted. The significance between the control and MitoC treated groups was calculated by the *t*-test.

2.9 *Serum half-life and extent of protein binding*

High-pressure liquid chromatography (HPLC) method was developed for quantitative determination of MitoC in mice serum. Prior to HPLC analysis, the protocol for MitoC extraction from serum was optimized. Standard stock solutions of MitoC of different concentrations 1000, 500, 250, 100, 50, 25, 10, 5 μg/mL were prepared. BALB/c mice serum (100 μL) was diluted with distilled water (80 μl), internal standard (Emodin; 1 μl) and mixed with MitoC solution (10 μL) to get a final MitoC concentration of 100, 50, 25, 10, 5, 2.5, 1 and 0.5 μg/ml. The mixtures were vortexed for 2 min followed by the addition of 600 μl of extraction reagent (95% ethyl acetate and 5% methanol). The resulting mixtures were vortexed for 20 min vigorously. Each mixture was further centrifuged at 5000 rpm for 20 min and the supernatant above the organic layer was collected and this was dried with nitrogen gas. The obtained dry powder was reconstituted in 100 μl of acetonitrile and samples were loaded onto an HPLC system connected with a diode array detector for quantification of MitoC for estimation of extraction yield on the reverse-phase column (C18 Phenomenex column 5 μM, 4.6 mm × 250 mm) using optimized mobile phase of 2% glacial acetic acid: acetonitrile (pH, 3) in 46:54 ratio. Chromatography was performed at room temperature under the isocratic condition at a flow rate of 1.0 mL/min using a diode array detector at 420 nm. The retention time of pure MitoC (50 μg/ml) on the HPLC column was observed to be about 17.4 minutes. The extraction protocol led to nearly 100% recovery of mitocurcumin from serum, as estimated from the area under the curves (AUC).

For estimation of mitocurcumin serum-stability and degree of plasma protein binding, it was incubated (50 μg/ml) with mice serum (100 μl) for different

times followed by separation on the reverse-phase column. The concentration of MitoC at different time points was estimated from the ratio of AUCs for MitoC extracted from the serum and that of pure MitoC.

3. Results

3.1 *MitoC rapidly and efficiently kills bacteria*

A time-kill kinetic assay was performed using 1X and 4X MIC of MitoC, and 4X MIC of ciprofloxacin. The 4X MIC ciprofloxacin completely kills *B. subtilis* bacteria in about 4 h (figure 1). MitoC displayed superior bactericidal properties. Bacteria appeared to be non-viable within 5 min of treatment with 4X MIC of MitoC. No viable bacterial cells were observed after 1 h treatment at lower exposures (1X MIC) of MitoC (figure 1). The time-kill assay thus clearly suggests rapid and efficient bactericidal action of MitoC against *B. subtilis*.

3.2 *MitoC does not develop resistance in in vitro assay*

To assess the potential for the development of resistance against MitoC, a serial passage resistance selection experiment was performed. The initial MICs of MitoC and ciprofloxacin (as control) were determined for *S. carnosus* via the broth microdilution method and were found to be 1.5 μg/mL and 62 ng/mL respectively. Bacteria were then sub-cultured for several generations to determine resistance development in the form of an increase in MIC. Minimum inhibitory concentration for mitocurcumin changed marginally from 1.5 μg/ml to 3 μg/ml and did not increase further, suggesting no resistance was developed against mitocurcumin (figure 2). In comparison, MIC against ciprofloxacin increased 16-fold during the same period. Ciprofloxacin-resistant colonies did not show a decrease in resistance even after passing through antibiotic-free media for 10 generations. Notably, ciprofloxacin-resistant bacterial cells remain sensitive to the mitocurcumin with no change in MIC values.

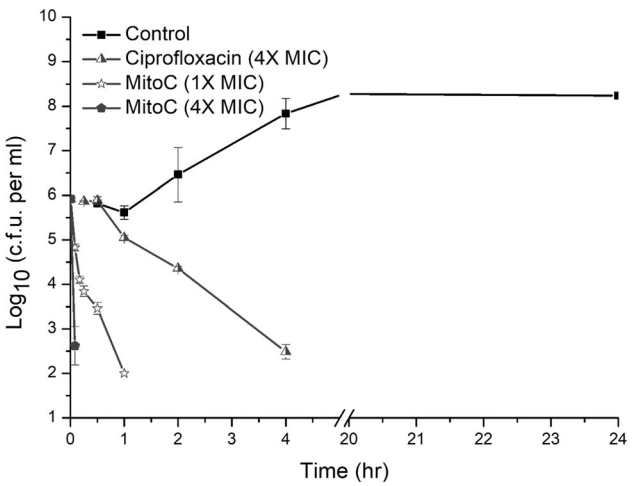

Figure 1. Time-kill curves of ciprofloxacin and mitocurcumin against *B. subtilis*. The bacteria (5×10^5 cells/ml) were cultured in MHB medium containing no antibiotic (control), 4-fold concentrations of the MIC of ciprofloxacin [ciprofloxacin (4X MIC)], MIC and 4-fold MIC of mitocurcumin [MitoC (1X MIC and 4X MIC)]. The viable count was performed at different time intervals using 10 μl aliquots, followed by serial microdilution in sterile MHB. All data points represent mean ± SE of three independent experiments.

3.3 *Antibacterial activity of mitocurcumin against multi-drug resistant pathogens*

Minimum inhibitory concentrations of MitoC were determined against four multi-drug resistance human pathogens (MRSA and MDR clinical *S. aureus* isolates and two clinically isolated *S. pneumoniae* strains) using the NCCLS-recommended broth microdilution method. Mitocurcumin displays MIC of 1.6 μg/ml against *S. aureus* strains and 12.5 μg/ml against *S. pneumonia*

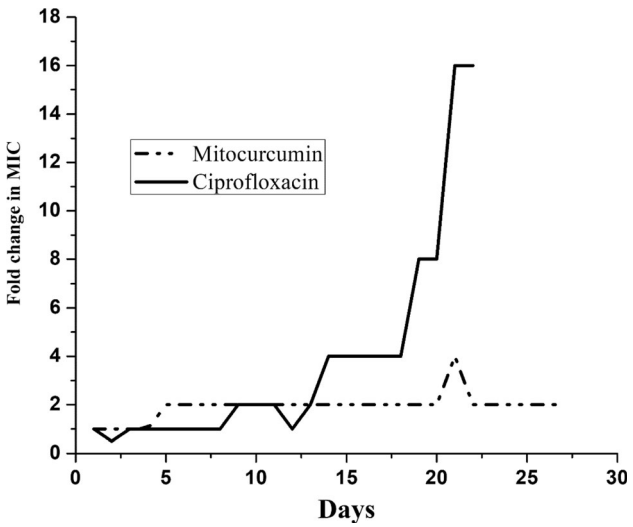

Figure 2. *In vitro* resistance development serial passage assay. Ciprofloxacin MIC against *S. carnosus* displayed increasing-trend from 14th day and increased to 16-fold after 23 generations. In comparison, MitoC MIC increased to only two-fold on 5th day and thereafter remained same throughout the period of this experiment.

strains, which can be considered highly promising given the resistance characteristics of these species against a variety of commonly used antibiotics. Antibiotic susceptibility profiles of the four tested bacterial species are given in supplementary table 1.

3.4 *TolC deletion does not affect MIC of E. coli*

Mitocurcumin displays higher MIC and MBC against Gram-negative *E. coli*, compared to Gram-positive bacteria (Kumari *et al*. 2019). The two bacteria differ markedly in the structure of outer-membrane and for the presence of efflux pumps. TolC is one of the outer-membrane efflux proteins that is a major player for multidrug efflux across the outer membrane of *E. coli* (Tikhonova and Zgurskaya 2004). The role of TolC mediated efflux pump was probed by determining MitoC activity against TolC deletion mutant of *E. coli*. Identical MIC was observed against wild-type and TolC deletion mutant, suggesting that TolC mediated efflux may not be the reason for higher MIC against *E. coli*.

3.5 *MitoC induces a concentration-dependent leakage of entrapped CFL from liposomes mimicking different cell types*

The effect of MitoC treatment on membrane stability was studied using CFL encapsulated liposome models mimicking eukaryotic and Gram-negative membranes at 37°C. Different MitoC concentrations were used for the assay with 10 min or 2 h treatment time. Leakage of CFL dye was estimated and disruption efficacy of MitoC was estimated from formula 1 against different membrane models.

Eukaryotic cell membrane mimicking liposomes showed less sensitivity to mitocurcumin, compared to the model for the Gram-negative membrane. The maximum impairment of about 61% was observed for MitoC concentration of 20 μM, while at 10 μM the disruption efficacy of about 14% was observed (figure 3) for eukaryotic membrane models. This compares favorably with the observed value of ∼12% death of human PBMNC treated with 10 μM of MitoC (Kumari *et al*. 2019). In contrast, nearly complete disruption efficacy was observed for 3 μM MitoC for the artificial membrane mimicking Gram-negative bacteria (figure 3). The important finding of this study was also the observation that the Gram-negative membrane was disrupted efficiently and rapidly: 5 μM of MitoC was sufficient to disrupt nearly 96% of Gram-negative liposomes in 10 min. Each of the assays was performed in triplicate and relative standard error was observed to be less than 1.5% in each of the independent studies. The artificial membrane for Gram-positive cells, however, was not stable and showed large leakage of fluorescent dye.

Table 1. Haematological parameters observed after 48 h of treatment with MitoC oral dose of 250 mg/kg body weight

Parameter	Control	MitoC
Hemoglobin (gm %)	13.3 ± 0.2	13.7 ± 0.3
Red Blood Cell (× 10^6/cmm)	7.7 ± 0.4	7.8 ± 0.2
White Blood Cell (× 10^3/cmm)	5.6 ± 0.4	5.9 ± 1.1
Platelets (× 10^5/cmm)	503.7 ± 45.5	556.3 ± 20.4
Packed Cell Volume (%)	38.9 ± 2.1	30.6 ± 7.9
Mean Corpuscular Volume (fl)	50.4 ± 0.8	51.0 ± 0.2
Mean Corpuscular Hemoglobin (pg)	17.3 ± 0.9	17.6 ± 0.4
Mean Corpuscular Hemoglobin Concentration (gm/dl)	34.4 ± 1.5	34.6 ± 0.8
Heterophile (%)	64.0 ± 4.0	58.3 ± 2.6
Eosinophils (%)	2.7 ± 1.2	2.3 ± 1.2
Lymphocytes (%)	32.3 ± 5.2	39.0 ± 3.5
Monocytes (%)	1 ± 0	0.3 ± 0.3

Values given are arithmetic mean ± standard error.

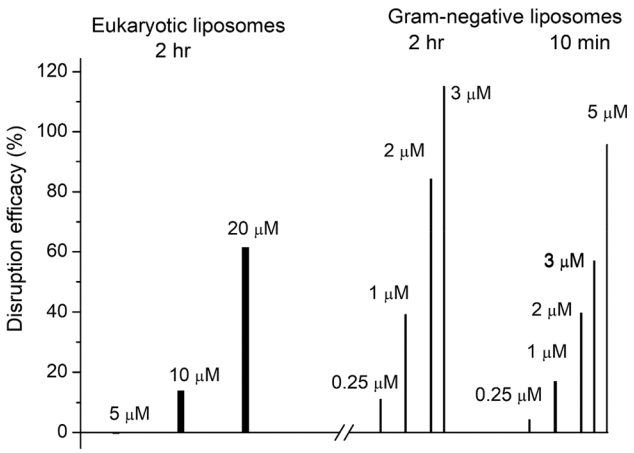

Figure 3. Liposomal disruption assay. The reconstituted liposomal membranes representing eukaryotic and Gram-negative cells were treated with varying MitoC concentration for 2 h and 10 min. The released CFL dye was monitored and disruption efficacy was estimated by equation 1. MitoC concentrations are shown above each data bar. Each of the assay was performed in triplicate and relative standard error were observed to be less than 1.5% in each of the independent study.

Table 2. Biochemical parameters observed after 48 h of treatment with MitoC oral dose of 250 mg/kg body mass

Parameter	Control (DMSO)	MitoC	CPCSEA reference (#)
Creatinine (mg/dl)	0.36 ± 0.02	0.36 ± 0.02	0.3-1
Alanine aminotransferase (u/l)	78.5 ± 14.50	39.3 ± 4.0	53 ± 14
Total Protein (g/dl)	6.2 ± 0.18	5.7 ± 0.14	3.5-7.2
Cholesterol (mg/dl)	70.6 ± 1.16	61.2 ± 3.80	26-82
Glucose (mg/dl)	61.8 ± 2.86	69.0 ± 2.02	62-175
Alkaline Phosphatase (u/l)	93.5 ± 5.50	156.0 ± 11.0	132 ± 20

Values given are arithmetic mean ± standard error.

Biochemical data of commonly used laboratory animals from the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA).

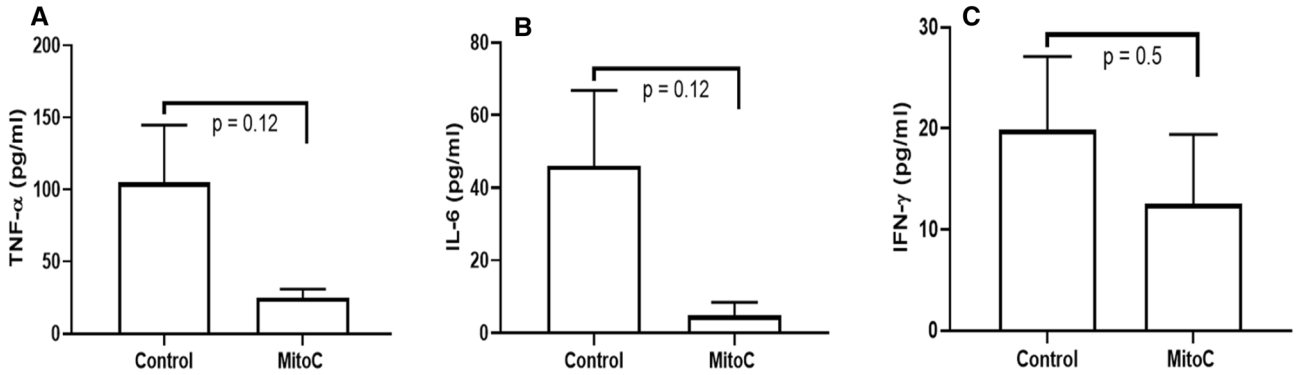

Figure 4. Effect of MitoC on the serum cytokines- TNF-α (**A**) IL-6 (**B**) and IFN-γ (**C**). The BALB/c mice were administered with MitoC (250 mg/kg body weight; oral), serum was isolated 24 hr after the treatment and cytokines were estimated using ELISA method. Three mice were used per group and the mean ± SD obtained from these three mice have been plotted. P value obtained from t-test also is indicated. However, the observed values are in the normal range reported in the literature [TNF-α, 24.6 pg/ml (10-250); IL-6, 2.6 pg/ml (5-240) and IFN-γ, 12.5 pg/ml (10-400)].

3.6 *Acute toxicity of MitoC in BALB/c mice*

When administered to mice via oral gavage, MitoC at a dose of 25 or 50 or 100 or 250 mg/kg body weight did not show any toxicity during the observation period of 10 days. In another experiment, when MitoC was administered through intraperitoneal route, all the mice survived up to a dose of 5 mg/kg body weight without any signs of toxicity.

3.7 *Effect of MitoC on haematological, biochemical and cytokines profiles*

Different haematological parameters were analysed by performing complete blood count on control and MitoC (oral dose of 250 mg/kg body weight) administered mice. Mice that were administered with MitoC did not show major change in haematological and biochemistry parameters, compared to the control group, except for the decrease in Alanine aminotransferase (ALT) and increase in alkaline phosphatase (ALP) (tables 1 and 2).

In order to assess the effect of MitoC on immune homeostasis in BALB/c mice, the levels of pro-inflammatory cytokines were measured. In both control and MitoC (250 mg/kg body weight) administered mice, levels of serum cytokines such as TNF-α, IL-6 and IFN-γ were measured by ELISA. The changes in the cytokine levels (TNF-α, IL-6 and IFN-γ) are seemingly very close to the normal range in MitoC treated group as compared to vehicle-treated group but they are not statistically significant (figure 4). The observed values were within the normal ranges (10–250 pg/ml, 5–240 pg/ml and 10–400 pg/ml, for TNF-α, IL-6 and IFN-γ respectively) as reported in the literature (Stenina *et al*. 2012).

3.8 *Serum half-life and amount of unbound drug*

Concentrations of free drug in serum are taken as good predictors of the time course of unbound drug in

interstitial fluid and may manifest in determining *in vivo* efficacy. We estimated MitoC serum stability by HPLC assay. Mitocurcumin was found to be highly stable in the serum of experimental mice. For instance, nearly 93% of MitoC was recovered after incubation with mice serum for 120 min (supplementary figure 1).

4. Discussion

Increasing resistance against available antibiotics in the pathogenic bacteria is a cause of serious concern world-over. The World Health Organization (WHO) has recently listed a catalogue of 12 families of antibiotic-resistant bacteria that pose the greatest threat to human health and for which new antibiotics are urgently needed. Molecules structurally distant from known antibiotics and possessing membrane targeting bactericidal activities are expected to display activity against drug-resistant pathogens. A structurally distinct molecule has recently been identified using an Artificial Intelligence approach that displays strong antibiotic activity (Stokes *et al*. 2020).

We have recently characterized a structurally novel derivative of curcumin, mitocurcumin (MitoC), which displays broad-spectrum bactericidal activity (Kumari *et al*. 2019). We reported static *in vitro* MIC values of about 2 μg/ml against Gram-positive bacteria and 10 μg/ml against laboratory *E. coli* strain.

In the present study, we report rapid and efficient bactericidal action of mitocurcumin against *B. subtilis* by the time-kill assay. The bacteria were rendered totally non-viable within 5 min of exposure to MitoC at 4X MIC. Interestingly, coagulase-negative *S. carnosus* did not develop resistance to MitoC and it also efficiently killed ciprofloxacin-resistant bacterial population experimentally generated in the laboratory.

Use of lipid models for simulating cytoplasmic membranes of bacteria is being intensely debated as the synthetic membranes lack membrane potential – a hallmark feature of bacterial membranes compared to the eukaryotic cell wall. We find here good consistency of MitoC activity towards eukaryotic and prokaryotic lipid models and cell types (figure 3). MitoC displays MIC of 10 μM against *E. coli* cells and affects the Gram-negative liposomal mimic severely, with 3 μM of the agent sufficient to completely impair the model membrane in 2 h. However, only 10% of the human PBMNC were detected to be dead in 24 h treatment and eukaryotic liposomal disruption efficacy of about 14% is observed with 10 μM of MitoC in 2 h. Interestingly also, the same MIC values (10 μM) were observed for *E. coli* cells with or without the outer-membrane TolC efflux pump. The combination of results on lipid membranes and the TolC deletion mutant provides a reasonable understanding of MitoC bactericidal activity and are suggestive of primary action on the inner plasma (cytoplasmic) membrane of the bacterium. This study is thus useful for further developing it as a therapeutic molecule.

In view of the dual and often conflicting effects of protein-binding of antibiotics (such as reducing free drug molecule concentration and/or reducing drug clearance), testing the impact of protein binding *in vitro* has been suggested (Zeitlinger *et al*. 2011). We observed more than 93% of the mitocurcumin in the native and unbound state. This should lead to high bioavailability at the site of infection if it is not rapidly eliminated by the clearance mechanisms of the host.

Another notable outcome of the present study was the finding that mitocurcumin displays MIC of 1.6 μg/ml against methicillin-resistant and clinical *S. aureus* isolates, and 12.5 μg/ml against multi-drug-resistant clinical *S. pneumonia* strains *in vitro*. These pathogens are regarded by the WHO as the 'priority pathogens' that pose the greatest threat to human health (*https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed*) and most urgently require new treatments. The MIC value of MitoC against drug-resistant *S. aureus* is about 5 times lower than the non-toxic concentration observed earlier for different types of human cells (Kumari *et al*. 2019). Furthermore, 5 mg/kg body weight of intraperitoneally administered MitoC (equivalent to ∼25 μM levels in the blood) was observed to be totally safe, suggesting the clinical potential of the agent against otherwise resistant pathogens.

We observe a safe oral dose of 250 mg/kg body weight in the murine model. This dose did not cause mortality or morbidity in the subjected rodents. A single safe oral dose also did not significantly alter haematology or biochemistry parameters within 48 h, except for the decrease in Alanine aminotransferase (ALT) and increase in alkaline phosphatase (ALP) (tables 1 and 2). Both these biochemistry parameters are related to liver function, but are within the permissible range of biochemical data given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Earlier, ALP has also been demonstrated to be a reliable marker to accurately assess cholestasis in mice (Krones *et al*. 2015). Mitocurcumin, however, reduced serum cytokine levels (TNF-α, IL-6 and IFN-γ), with the maximum influence on

pro-inflammatory cytokine IL-6 (figure 4). The levels observed for TNF-α and IFN-γ in MitoC treated group fall in the normal reported range. However, levels of pro-inflammatory cytokine IL-6 was lower than the reported normal range. High levels of IL-6 are known to cause cytokine storm which can lead to multi-organ failure. Reduction in the levels of IL-6 after MitoC administration may reduce the possibility of a cytokine storm. To understand whether these changes in the cytokines may have positive outcomes during infection or any possible unwanted side effects in the host-system warrants further detailed studies.

Given the excellent antibacterial profile of mitocurcumin, especially against the WHO recognized antibiotic-resistant bacterial species, we observe that it is a highly promising lead molecule.

5. Conclusions

We report here the pharmacological characterization of mitocurcumin that displays wide-spectrum bactericidal activity, including activity against multidrug-resistant clinical isolates. The safe oral dose does not significantly alter haematological and biochemical parameters and inflammatory responses in the mice model. Mitocurcumin does not show resistance development against *S. carnosus* and ciprofloxacin-resistant bacterial cells remain sensitive to mitocurcumin. Rapid bactericidal action, as assessed by *in vitro* time-kill assay and liposomal membrane models, and long half-life in rodent serum strongly suggest that MitoC is an excellent lead molecule, especially against drug-resistant pathogens.

Acknowledgements

The authors would like to thank Dr. Mahesh Subramanian for providing *E. coli* TolC deletion mutant and Ms. Binita Kisley for technical help in experiments related to HPLC.

Funding

The research was funded by the Department of Atomic Energy, India.

Ethics approval

BALB/c mice were used in the study under the approval of Institutional Animal Ethics Committee granted for the Project No. BAEC/29/19.

References

Alkharfy KM, Kellum JA, Frye RF and Matzke GR 2000 Effect of ceftazidime on systemic cytokine concentrations in rats. *Antimicrob. Agents Chemother.* **44** 3217–3219

Epand RF, Savage PB and Epand RM 2007 Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). *Biochim. Biophys. Acta* **1768** 2500–2509

Frieling JT, Mulder JA, Hendriks T, Curfs JH, van der Linden CJ and Sauerwein RW 1997 Differential induction of pro- and anti-inflammatory cytokines in whole blood by bacteria: effects of antibiotic treatment. *Antimicrob. Agents Chemother.* **41** 1439–1443

Huggins MA, Sjaastad FV, Pierson M *et al*. 2019 Microbial exposure enhances immunity to pathogens recognized by TLR2 but increases susceptibility to cytokine storm through TLR4 sensitization. *Cell Rep.* **28** 1729–1743.e5

Jayakumar S, Patwardhan RS, Pal D, Singh B, Sharma D, Kutala VK and Sandur SK 2017 Mitochondrial targeted curcumin exhibits anticancer effects through disruption of mitochondrial redox and modulation of TrxR2 activity. *Free Radic. Biol. Med.* **113** 530–538

Kohanski MA, Dwyer DJ and Collins JJ 2010 How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8** 423–435

Krones E, Erwa W, Trauner M and Fickert P 2015 Serum alkaline phosphatase levels accurately reflect cholestasis in mice. *Hepatology* **62** 981–983

Kumari S, Jayakumar S, Gupta GD, Bihani SC, Sharma D, Kutala VK, Sandur SK and Kumar V 2019 Antibacterial activity of new structural class of semisynthetic molecule, triphenyl-phosphonium conjugated diarylheptanoid. *Free Radic. Biol. Med.* **143** 140–145

Li J, Koh JJ, Liu S, Lakshminarayanan R, Verma CS and Beuerman RW 2017 Membrane active antimicrobial peptides: translating mechanistic insights to design. *Front. Neurosci.* **11** 73

Melhus A 2001 Effects of amoxicillin on the expression of cytokines during experimental acute otitis media caused by non-typeable Haemophilus influenzae. *J. Antimicrob. Chemother.* **48** 397–402

Mueller M, de la Pena A and Derendorf H 2004 Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. *Antimicrob. Agents. Chemother.* **48** 369–377

Nielsen EI, Viberg A, Lowdin E, Cars O, Karlsson MO and Sandstrom M 2007 Semimechanistic pharmacokinetic/pharmacodynamic model for assessment of activity of antibacterial agents from time-kill curve experiments. *Antimicrob. Agents Chemother.* **51** 128–136

Pinheiro M, Magalhaes J and Reis S 2019 Antibiotic interactions using liposomes as model lipid membranes. *Chem. Phys. Lipids* **222** 36–46

Stenina MA, Krivov LI, Voevodin DA, Savchuk VI, Kovalchuk LV and Yarygin VN 2012 Cytokine profile of the blood in mice with normal and abnormal heart rhythm. *Bull. Exp. Biol. Med.* **196** 692–695

Stokes JM, Yang K, Swanson K, *et al*. 2020 A deep learning approach to antibiotic discovery. *Cell* **181** 475–483

Tikhonova EB and Zgurskaya HI 2004 AcrA, AcrB, and TolC of *Escherichia coli* form a stable intermembrane multidrug efflux complex. *J. Biol. Chem.* **279** 32116–32124

Zeitlinger MA, Derendorf H, Mouton JW, Cars O, Craig WA, Andes D and Theuretzbacher U 2011 Protein binding: do we ever learn? *Antimicrob. Agents Chemother.* **55** 3067–3074

Zhang H 2017 Thin-Film Hydration Followed by extrusion method for liposome preparation. *Methods Mol. Biol.* **1522** 17–22

Corresponding editor: BJ RAO