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Antimycobacterial activity of bacteriocins and their complexes with liposomes

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***Objectives*: Bacteriocins (Bcn) are natural peptides that are secreted by several taxonomically distant bacteria and exert bactericidal activity against other bacterial species. Their capacity to inhibit growth of virulent Mycobacterium tuberculosis H37Rv was evaluated in this study.**

***Methods*: Five different Bcn were isolated and purified from bacterial culture supernatants, their amino acid sequence was determined, and activity against mycobacteria assessed in three different models: *in vitro* mycobacterial cultures, *in vitro* infection of mouse macrophages and *in vivo* high-dose infection of inbred mice.**

***Results*: In the *in vitro* model, four out of five Bcn exhibited stronger antimycobacterial activity than equal concentrations of a widely used anti-TB antibiotic, rifampicin. These Bcn were non-toxic for mouse macrophages at a concentration of 0.1 mg/L (>MIC_{90} of these compounds). Pure Bcn did not inhibit mycobacterial growth within murine macrophages when added at 0.01–0.1 mg/L, suggesting that at physiologically tolerable concentrations these molecules do not penetrate through the membrane of eukaryotic cells. However, when administered as a complex with phosphatidylcholine–cardiolipin liposomes, Bcn5 (selected as a model compound due to its cytotoxicity and antimycobacterial activity regular titration curves) demonstrated capacity both to inhibit intracellular growth of *M. tuberculosis* and to prolong survival of mice in an acute TB model.**

***Conclusions*: Given that the mechanism of Bcn bactericidal activity differs from that of all commonly used antibiotics, their possible involvement in complex TB therapies deserves further study.**

Keywords: tuberculosis chemotherapy, mouse model, *Mycobacterium tuberculosis*

Introduction

According to the WHO, about one-third of the world's population is latently infected with *Mycobacterium tuberculosis*, and from this pool, roughly 9 million cases of active tuberculosis (TB) emerge annually, resulting in 2–3 million deaths.[1] The two main current strategies to control TB are drug treatment of active disease cases and BCG vaccination to protect children. Given that the efficacy of current first-line anti-TB drugs is often reduced because of mycobacterial drug resistance[2,3] and that the performance of the only available BCG vaccine is poor in the populations most at risk,[4] there is a general consensus that the development of new therapies and vaccines against TB is a global challenge. Thus, it is not surprising that many institutions and laboratories are aiming to stop the spread of TB by developing new faster-acting and affordable TB drugs effective against multidrug-resistant (MDR) mycobacterial strains.[3,5]

It has been reported recently that it is possible to develop new anti-TB therapies by chemical optimization of antimycobacterial prototype compounds, such as diarylquinolines and 1,2-ethylenediamines, and that some of the new drugs show a better performance than currently available agents and are

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effective against MDR mycobacterial strains both *in vitro* and in animal TB models.[6–8] Among these new drugs, the R207910 compound is especially attractive for a rapid introduction in clinical practice: its spectrum is unique in selective specificity to mycobacteria, there is no cross-resistance with currently used anti-TB remedies and the target and mechanism of action (inhibition of ATP synthase function) are different from those of other TB drugs. Nevertheless, the search for antimycobacterial agents with distinct mechanisms of action should be continued.[6]

It is well established that a specific class of peptides derived from diverse Gram-positive bacteria species—bacteriocins (Bcn)—exert activity against a wide range of bacteria. Bcn produced by lactic acid bacteria are categorized into three classes, and the mode of action of class I and IIa Bcn has been thoroughly studied.[9,10] For the vast majority of these Bcn, a membrane-permeabilizing mode of action inducing leakage of low-molecular-weight compounds from target cells has been shown.[9–12] The chemical structure and the mode of action of Bcn suggest that neither common genetic mechanism of mycobacteria leading to MDR, such as mutations in *rpoB*, *katG–inhA*, *embA-C*, nor quinolone-resistance mutations in *gyrA* and *gyrB* genes[13] will be effective against this class of biologically active molecules. Here, we report the results of our experiments on evaluation of the efficacy of five different class IIa-like Bcn against *M. tuberculosis* H37Rv in three different models: *in vitro* mycobacterial cultures, *in vitro* infection of mouse macrophages and *in vivo* infection of inbred mice.

Materials and methods

Bacterial source of bacteriocins

Five different bacteriocins [B37 (hereafter Bcn1), B602 (Bcn2), OR7 (Bcn3), S760 (Bcn4) and E50-52 (Bcn5)] were obtained from isolates of different bacterial species. Preparation of Bcn1 and Bcn2 has been described previously.[14] After cloning and re-cloning, candidate isolates that served as the source of the three other bacteriocins were morphologically identified as *Lactobacillus salivarius* (Bcn3), *Streptococcus cricetus* (Bcn4) and *Enterococcus faecalis* (Bcn5), according to the API 50 CH typing system.

Production of crude antimicrobial preparations

Bacteria were grown for 40 h at 37°C under aerobic conditions in 250 mL of modified Kugler broth medium for antimicrobial susceptibility testing of *Haemophilus influenzae*[15] supplemented with alanine, tryptophan and glucose. The stationary cultures were centrifuged at 2500 **g** for 10 min to remove viable cells. The decanted supernatants were mixed with 80% saturated ammonium sulphate and incubated at 4°C for 24 h to precipitate protein compounds. Following centrifugation at 10 000 **g** for 20 min, the sediment was resuspended in 1.5 mL of 10 mM PBS (pH 7.0) and dialysed overnight against 2.5 L of buffer. The solution obtained was designated 'crude antimicrobial preparation (CAP)'. Each CAP was filtered through a 0.22 μm pore filter (Millipore, Bedford, MA, USA).

Purification of bacteriocins

Bacteriocins were purified from CAP by gel filtration on 50 cm Superose 12HR 16/50 columns (Pharmacia, Uppsala, Sweden), and eluted fractions were tested against three *Campylobacter jejuni* strains using the spot test as previously described.[14] Fractions demonstrating antimicrobial activity were further purified on 20 cm Mono Q HR 5/5 columns and on 20 cm CM-Sepharose columns (Pharmacia). Antimicrobial activity and protein concentration were determined for each fraction, molecular weights of fractions were determined by electrophoresis and individual cuts from strips containing bacteriocins were further tested against three target *C. jejuni* isolates and *C. jejuni* strain NCTC 11168 by a method described previously.[16] Molecular masses of bacteriocins were confirmed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry, and amino acid sequences of bacteriocins were determined by Edman degradation using a 491 cLC Automatic Sequencer (Applied Biosystems, La Jolla, CA, USA), according to the manufacturer's instructions. All five isolated Bcn contained the amino-terminal sequence motif YGNGV shared by all class IIa bacteriocins (Table 1).

Preparation of bacteriocin–liposome complexes

Soy phosphatidylcholine was obtained from Lipoid (Germany) and bovine cardiolipin was obtained from Biolek (Kharkov, Ukraine). Twenty milligrams of a 1:4 mixture of phosphatidylcholine (Ph) and cardiolipin (C) was dissolved in ethanol and evaporated under vacuum conditions. The lipid film was resuspended in 1 mL of saline and Bcn5 at a concentration of 10 mg/mL was added in a 100 μL volume with gentle stirring. PhC–Bcn5 complex was separated from free Bcn5 and liposomes by centrifugation at 5000 **g** for 30 min. The pellet was dispersed in 0.5 mL of saline, and the resulting liposomes with an average diameter of <150 nm were isolated by extrusion using a LiposoFast Basic extruder (Avestin Inc., Canada). Concentrations of Bcn5 and lipids in the preparation were

Table 1. Amino acid sequence and sequence alignment of bacteriocins

Bacteriocin	Amino acid sequence[a]	Molecular mass (Da)[b]	pI
Bcn1	FVYGNGVTSILVQAQFLVNGQRRFFYTPDK	3214	4.8
Bcn2	ATYYGNGLYCNKQKHYTWVDWNKASREIGKITVNGWVQH	3864	7.2
Bcn3	KTYYGTNGVHCTKNSLWGKVRLKNMKYDQNTTYMGRLQDILLGWATGAFGKTH	5123	8.4
Bcn4	RWYYGNGVGGVGGAAVCGLAGYVGEAKENIAGEVRKGWGMAGGFTHNKACKSFPGSGWASG	5362	9.1
Bcn5	TTKNYGNGVCNSVNWCQCGNVWASCNLATGCAAWLCKLA	3932	7.8

[a]Underlined residues are conserved by at least 70%.
[b]As determined by MALDI–TOF mass spectrometry.

measured by the modified Lowry method[17] and by the method described by Svetashev and Vaskovsky,[18] respectively.

Bacteria and animals

M. tuberculosis strain H37Rv (original stock—a gift from G. Marshal, Institute Pasteur, Paris, France) was maintained and prepared for macrophage *in vitro* infection exactly as described previously.[19] Briefly, the bulk stock of bacteria was stored at −80°C in 1 mL aliquots containing 10^8 mycobacterial cfu in sterile saline supplemented with 0.05% Tween 20 and 0.1% BSA (Sigma, St Louis, MO, USA). To obtain the log-phase bacteria for macrophage challenge, 50 μL from a thawed aliquot was added to 5 mL of Dubos broth (Difco, Detroit, MI, USA) supplemented with 0.5% BSA (Sigma) and incubated for 1 week at 37°C. A volume of 0.5 mL of mycobacterial suspension was diluted in 20 mL of fresh warm Dubos/BSA medium and further cultured for 1 week. The resulting suspension was washed three times at 3000 **g**, 4°C, with 0.02% EDTA–PBS (Ca^{2+}, Mg^{2+} free) solution, resuspended in supplemented, antibiotic-free cell culture medium RPMI-1640 (HyClone, Carlington, The Netherlands) and filtered through a 4 μm pore size filter (Sigma) to remove clumps. To estimate the cfu content in filtrate, 10 μL from each of 5-fold serial dilutions was plated onto Dubos agar (Difco), and the total number of microcolonies in the spot was calculated under inverted microscope after culturing for 3 days at 37°C. The bulk of the filtered culture was stored at +4°C, and it was found that no change in the cfu content occurred during this period.

C57BL/6JCit (B6) mice were bred under conventional conditions at the animal facilities of the Central Institute for Tuberculosis (Moscow, Russia, US Office of Laboratory Animal Welfare assurance A5502-01) in accordance with guideline no. 755 from the Russian Ministry of Health. Water and food were provided *ad libitum*. Female mice 10 weeks of age were used. Mice were challenged with mycobacteria from the bulk storage at a dose of 2×10^7 cfu/mouse in a 0.2 mL volume via the lateral tail vein. This resulted in a rapidly progressing lethal TB infection, as described previously.[20] To assess the efficacy of treatment, 10 μg/mouse of Bcn5 or 75 μg/mouse of rifampicin was given daily for 5 consecutive days starting 6 h post-challenge via intravenous (iv) route. Mortality was monitored daily. All experimental procedures were approved by the institutional animal care and use committee (IACUC).

Peritoneal macrophage isolation

Peritoneal macrophage isolation was performed exactly, as described previously.[19] Briefly, B6 mice were injected intraperitoneally with 3% peptone (Sigma) in saline. Five days later, peritoneal exudate cells were eluted from peritoneal cavities with Hanks balanced salt solution, supplemented with 2% fetal calf serum and 10 U/mL heparin. Cells were washed twice, resuspended in supplemented RPMI-1640 and 5×10^4 cells/well were allowed to adhere to the bottom of 96-well tissue culture plates (Costar-Corning, Badhoevedorp, The Netherlands) for 1.5 h before *in vitro* infection. Viability of macrophages, as determined by Trypan Blue exclusion, was >98%, and the content of non-specific esterase-positive cells was >92%.

Evaluation of mycobacterial growth

To assess the growth of mycobacteria in peritoneal macrophages, cell cultures were infected at a multiplicity of infection of 1:1. The growth of mycobacteria was assessed by [^{3}H]uracil uptake, as described previously.[19] Macrophages were allowed to adhere for 2 h and a viable single-cell mycobacterial suspension was added. The drugs—bacteriocins, their complexes with liposomes and rifampicin—were diluted (emulsified) in RPMI-1640 and added at indicated concentrations after the first 18 h of co-culture. After 24 h of incubation, 1 μCi/well [^{3}H]uracil (Isotop, St Petersburg, Russia) was added for the last 18 h of culture. The cultures were terminated by freezing the plates at −30°C, and harvest on fibreglass filters (Scatron, Norway) was performed after thawing the contents. The results ([^{3}H]uracil uptake by mycobacteria, cpm) were measured in triplicate in a liquid scintillation counter (Wallac, Finland). It was shown previously that parallel estimation of mycobacterial cfu counts and [^{3}H]uracil uptake under identical conditions provides >90% correlation,[19] validating the use of the latter less laborious and variable technique. An identical protocol, except plating macrophages, was used for the evaluation of the mycobacterial growth in macrophage-free cultures.

Lactate dehydrogenase release from macrophages

Lactate dehydrogenase (LDH) release from macrophages, as a measure of macrophage lysis, was determined by the enzymic activity of LDH in cultural supernatants using a CytoTox 96 kit (Promega, Madison, WI, USA), as recommended by the manufacturer. The percentage of specific lysis (SL) was calculated according to the formula: SL = [absorbance at 490 nm (A_{490}) in experimental wells − A_{490} after spontaneous release/A_{490} total lysis − A_{490} after spontaneous release] × 100.

Results and discussion

In the first set of experiments, we characterized the ability of bacteriocins to inhibit mycobacterial multiplication under conventional growth conditions in Dubos broth. To quantitatively

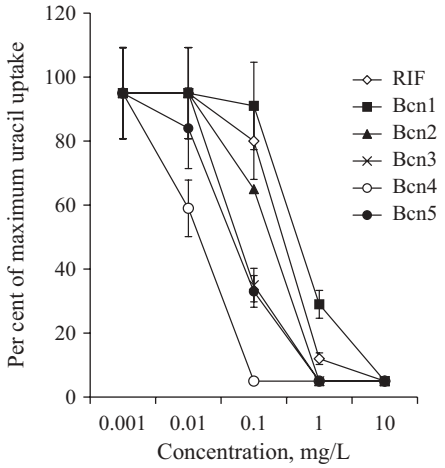

Figure 1. Inhibition of mycobacterial growth *in vitro* by Bcn. *M. tuberculosis* H37Rv (5×10^4 cfu/well of a 96-well flat-bottomed plate) was cultured in antibiotic-free RPMI-1640 at 37°C for 24 h and different Bcn were added at indicated concentrations, with rifampicin (RIF) used as a control drug. After an additional 24 h of incubation, [^{3}H]uracil (1 μCi/well) was added overnight, and the label uptake in triplicate wells was measured in a liquid scintillation counter. Results represent means ± SD of three independent experiments ($n = 9$ wells).

estimate metabolically active bacilli, we used a surrogate method based upon the uptake of [^{3}H]uracil, which proved to highly correlate with cfu counts.[19] As shown in Figure 1, Bcn2, 3, 4 and 5 inhibited mycobacterial growth more effectively than equal concentrations of the classical antimycobacterial drug rifampicin used as a control. Moreover, Bcn4 exhibited full bacteriostatic capacity at a concentration of 0.1 mg/L, thus demonstrating ~10-fold lower MIC_{50} and MIC_{90} than rifampicin.

Since the main mechanism of class IIa Bcn bactericidal action is formation of pores in cell membranes,[9,10] before testing their activity against macrophage-engulfed mycobacteria, it was important to find out whether or not these molecules are cytotoxic for the host macrophages and whether it is possible to find a range of concentrations bearable by the host cells but still effective against mycobacteria. To this end, we established *in vitro* cultures of peritoneal mouse macrophages and assessed their membrane integrity in the presence of serially diluted Bcn by a common LDH release assay. As shown in Figure 2, neither rifampicin nor Bcn1 (whose performance in bacteriostatic tests was the worse, see Figure 1) expressed cytotoxic activity even at the highest concentration tested (10 mg/L). Cytotoxicity of Bcn2 and 3 was negligible at a concentration of 1 mg/L and that of the two most potent (Bcn4 and 5) disappeared at a concentration of 0.1 mg/L. Importantly, these non-hazardous concentrations are within the MIC_{90} limits of corresponding compounds, providing rationale conditions for their further testing.

M. tuberculosis is a facultative intracellular parasite, and the vast majority of bacilli in an infected host reside within macrophages, cells that form the major niche for mycobacterial survival and multiplication and are also key to the effective control of the infection.[21] Thus, the ability to penetrate macrophage membranes and reach the mycobacterial target inside the host cell is a requisite feature of any antimycobacterial remedy. We anticipated that such big molecules as Bcn, with their molecular mass ranging between 3 and 6 kDa, would require an additional delivery system, e.g. liposome mixtures, to penetrate into macrophages and kill intracellular bacilli. Nevertheless, we tested the inhibitory activity of pure Bcn against mycobacteria engulfed by peritoneal mouse macrophages in an *in vitro* model. As shown in Table 2, in Bcn-treated macrophage–mycobacteria co-cultures, inhibition of mycobacterial growth fully depended upon the ability of Bcn at a given concentration to degrade macrophages themselves, as measured by the LDH release. Thus, Bcn1, which did not show any cytotoxic effect even at 10 mg/L (Figure 2), demonstrated only a marginal inhibitory effect on mycobacteria. In contrast, Bcn4, which was the most potent in an *in vitro* model (Figure 1), almost completely inhibited mycobacterial growth at its cytotoxic concentrations, but was ineffective at a non-cytotoxic concentration of 0.1 mg/L. These results indicate that, unlike rifampicin, which penetrates macrophage membrane without damaging it and kills intracellular mycobacteria in a concentration-dependent manner (Table 2), Bcn are effective only against mycobacteria released from disintegrated macrophages.

To facilitate Bcn delivery inside the host cells, we prepared a complex consisting of Bcn enmeshed into liposomes. Bcn5, as the compound whose cytotoxicity and antimycobacterial activity were characterized by regular titration curves (Figures 1 and 2), was used in further experiments. Depending on composition and concentration, liposomes themselves may either promote or inhibit mycobacterial growth, both in culture media and within macrophages, presumably due to the shifts in carbon metabolism that occur when external fats are abundantly present.[22] Thus, we pre-selected *in vitro* the type and concentration of a liposome

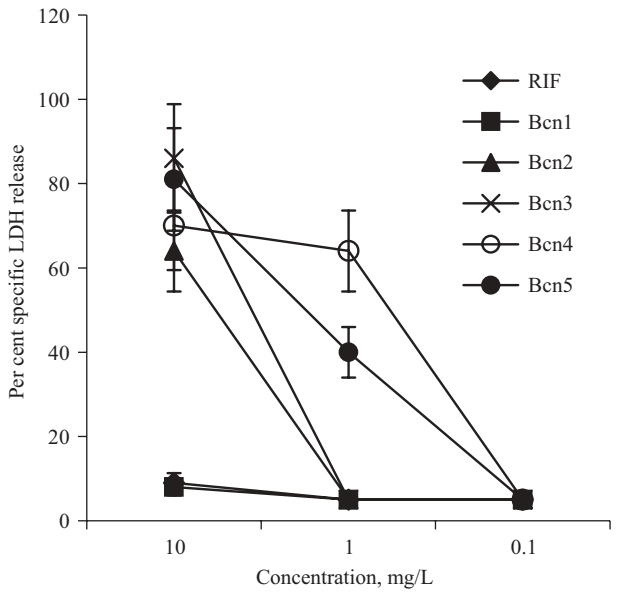

Figure 2. Cytopathic effect of Bcn on peritoneal mouse macrophages. Macrophages were isolated from peritoneal cavities and cultured at 5×10^4 per well in supplemented RPMI-1640 (see the Materials and methods section). On day 1 of culture, different Bcn or rifampicin (RIF) were added to wells at indicated concentrations, and specific LDH release was estimated after 48 h of incubation. Results (means for triplicate wells ± SD) of one out of three similar experiments are shown. No preparation displayed cytotoxicity at a concentration of 0.1 mg/L.

Table 2. Dependence between capacity of bacteriocins to inhibit mycobacterial growth and their cytotoxic activity against macrophages containing bacilli

Drug	Concentration (mg/L)	Per cent specific LDH release	Per cent maximum [^{3}H]uracil uptake
Rifampicin	10	9 ± 3	8 ± 5
	1.0	<5	45 ± 6
	0.1	<5	92 ± 5
Bcn1	10	8 ± 3	68 ± 4
	1.0	<5	88 ± 7
	0.1	<5	>95
Bcn2	10	64 ± 5	15 ± 2
	1.0	<5	76 ± 3
	0.1	<5	>95
Bcn3	10	86 ± 5	7 ± 2
	1.0	<5	69 ± 5
	0.1	<5	76 ± 6
Bcn4	10	70 ± 3	4 ± 1
	1.0	64 ± 4	12 ± 2
	0.1	<5	87 ± 8
Bcn5	10	81 ± 4	7 ± 2
	1.0	40 ± 6	35 ± 5
	0.1	<5	>95

that by itself has a minimal effect on mycobacterial growth, therefore not interfering with the evaluation of Bcn activity. Among five different types of liposomes tested (data not shown), liposomes consisting of a 1:4 phosphatidylcholine–cardiolipin mixture were chosen for further experiments. As shown in Figure 3(a), at a concentration of 7 mg/L, these liposomes neither altered mycobacterial growth when added to the medium alone nor demonstrated an inhibitory effect on mycobacteriostatic activity of Bcn5.

We then tested whether Bcn5–PhC complex: (i) is cytotoxic for murine macrophages; and (ii) can inhibit the growth of intracellular mycobacteria. Macrophages themselves were able to partially inhibit mycobacterial growth (~3.5-fold drop in [^{3}H]uracil uptake by mycobacteria when compared with macrophage-free cultures). Nevertheless, virulent mycobacteria displayed a marked cytopathic capacity (Figure 3b), since 48 h of infection significantly increased the proportion of macrophages that underwent membrane damage and released LDH in culture supernatant when compared with non-infected control cells ($38 \pm 6\%$ and $16 \pm 5\%$ of maximum LDH release, respectively, $P < 0.01$, Student's t-test). Importantly, treatment of infected cultures with Bcn5 alone, PhC liposomes

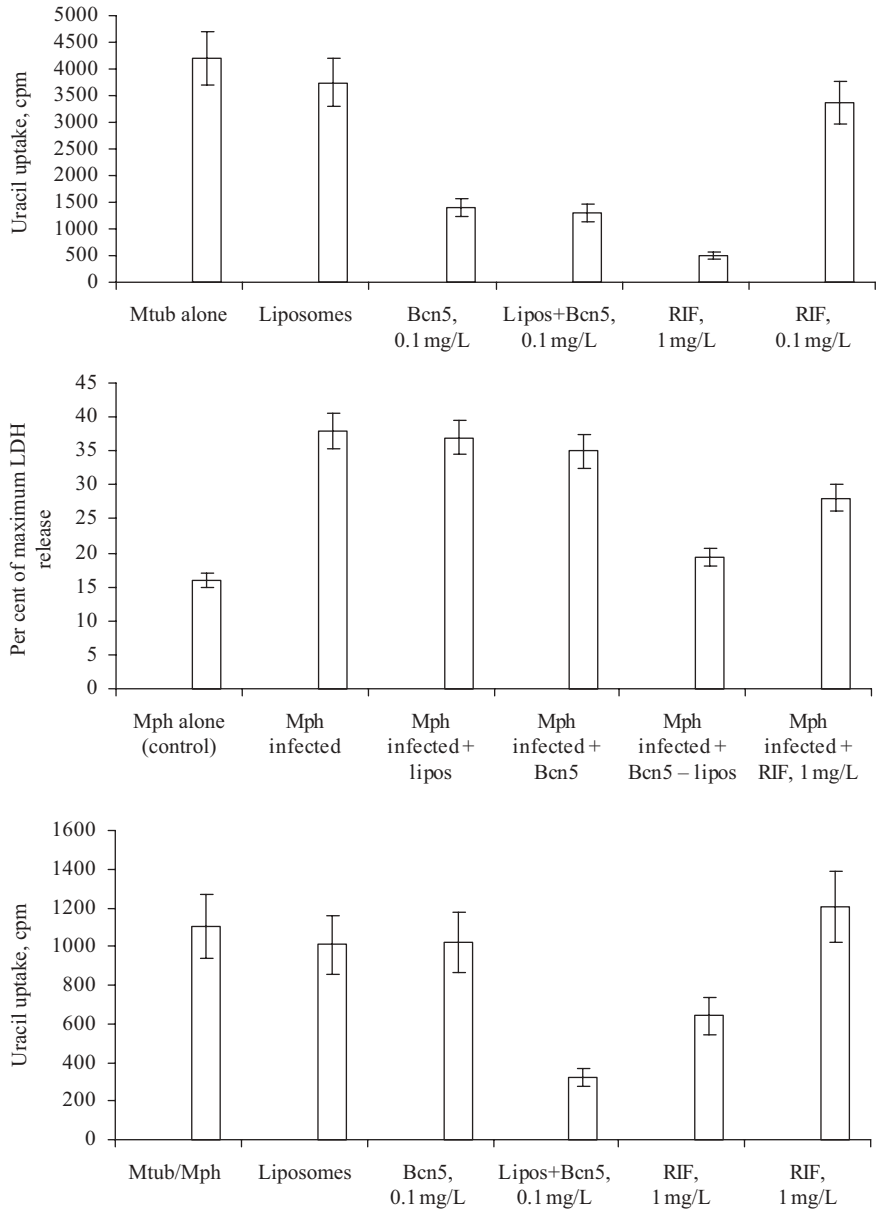

Figure 3. Bcn5–liposome complex is able to inhibit mycobacterial growth inside macrophages without damaging macrophage membrane. (a) Liposomes alone (7 mg/L) have no effect on mycobacterial growth and do not interfere with antimycobacterial activity of Bcn5 in macrophage-free cultures. (b) Infection with mycobacteria results in significant increase in LDH release from macrophages (Mph alone versus Mph infected); treatment with liposomes or Bcn5 causes no additional cell damage. (c) At a non-cytotoxic concentration (0.1 mg/L), Bcn5 alone has only a marginal effect on intracellular growth of mycobacteria, but significantly ($P < 0.01$, Student's t-test) inhibits their multiplication when administered in complex with liposomes. Results of one out of three (a and b) to five (c) similar experiments are displayed as means for triplicate wells $\pm$ SD. Mtub, *M. tuberculosis*; lipos, liposomes; Mph, macrophages; RIF, rifampicin.

Table 3. Influence of treatment on survival time following infection in a mouse model of acute lethal TB infection[a]

Experimental group	Mean survival time (MST) ± SD (days)
No treatment	21 ± 3
Bcn5 alone[b]	21 ± 3
PhC alone	21 ± 2
Bcn5–PhC complex[b]	28 ± 3*
RIF[c]	34 ± 6

[a]*M. tuberculosis* H37Rv 2×10^7 cfu/mouse iv.
[b]Treatment: 10 μg/mouse of Bcn5 iv per day for 5 consecutive days starting day 1 post-infection. $P < 0.05$ compared with control groups.
[c]Treatment: 75 μg/mouse of rifampicin (RIF) iv per day for 5 consecutive days starting day 1 post-infection.
*$P < 0.05$ (Gohan's criterion) compared with three control groups.

alone or Bcn5–PhC complexes caused no additional cell damage (Figure 3b), suggesting that the experimental system is suitable for testing the antimycobacterial activity of Bcn5–PhC complexes against intracellular bacteria. As shown in Figure 3(c), Bcn5–PhC complexes inhibited mycobacterial growth within macrophages at least 3-fold more when compared with control cultures ($P < 0.001$, Student's *t*-test) at a non-cytotoxic concentration of 0.1 mg/L and demonstrated a stronger antimycobacterial effect than rifampicin administered at a high concentration (1 mg/L). Thus, an appropriate combination of Bcn with liposomes provided a preparation that has a desirable antimycobacterial activity and does not cause cell damage.

We finally studied the *in vivo* efficacy of Bcn5–PhC complexes as an anti-TB therapy in mice, using a high-dose infection model of acute TB similar to that described by Nikonenko *et al.*,[23] which is convenient for a preliminary rapid *in vivo* screen. As shown in Table 3, a short-term treatment of mice with Bcn5–PhC (one iv injection every 24 h for 5 consecutive days) was sufficient to significantly ($P < 0.05$, Gohan's criterion for survival curves) increase the life span of infected animals. The effect was only slightly weaker than that of five injections with 10-fold higher concentrations of rifampicin (Table 3).

In conclusion, we showed in this study that class IIa Bcn produced by different bacteria constitute a set of molecules that are of great interest as potential anti-TB drugs, especially in combination with liposomal vectors conferring intracellular delivery. Given their natural origin, there is little doubt that the acquisition of highly specific resistance to Bcn by environmental microorganisms due to natural selection should follow the rules similar to those recently proposed for antibiotic resistance,[24] and, if includes mobile genetic elements, may be relatively rapidly transferred to human pathogens. Nevertheless, Bcn may serve as a good supplement to antibiotics in the treatment of MDR-TB since the mechanisms of action differ profoundly between these two classes of drugs and the development of cross-resistance is highly unlikely. Studies of Bcn efficacy against MDR mycobacterial strains in an aerosol mouse model of chronic TB are presently in progress.

Acknowledgements

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Transparency declarations

None to declare.

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