

## ORIGINAL ARTICLE

# Anti-*Mycobacterium* activity of microbial peptides in a silkworm infection model with *Mycobacterium smegmatis*

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An *in vivo*-mimic silkworm infection model with *Mycobacterium smegmatis* was established. When silkworms were raised at 37 °C following an injection of *M. smegmatis* cells ( $1.25 \times 10^7$  CFU larva<sup>-1</sup> g<sup>-1</sup>) into the silkworm hemolymph, they died within 48 h. Under these conditions, four microbial peptides with anti-*M. smegmatis* activity, lariatin A, calpinactam, lysocin E and propeptin, exerted therapeutic effects in a dose-dependent manner, and these are also clinically used agents that are active against *Mycobacterium tuberculosis*. These results indicate that the silkworm infection model with *M. smegmatis* is practically useful for the screening of therapeutically effective anti-*M. tuberculosis* antibiotics.

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## INTRODUCTION

In the process of antibiotic discovery, candidate compounds active against pathogenic microorganisms in an *in vitro* assay system often have no therapeutic effects in *in vivo* animal infection models. In order to overcome this issue, the therapeutic efficacies of candidate compounds need to be evaluated in an *in vivo*-mimic assay at the early stage of drug development. In our previous studies, a silkworm infection model with methicillin-resistant *Staphylococcus aureus* (MRSA) was established for the primary screening of anti-MRSA antibiotics, and led to the discovery of the new antibiotics, nosokomyins and lysocins.<sup>1–4</sup> Nosokomyin A and lysocin E exerted therapeutic effects in a mouse infection model with MRSA.<sup>1</sup> This silkworm infection was found to be applicable to other pathogenic microorganisms such as *Pseudomonas aeruginosa* and *Candida albicans*.<sup>5–8</sup> The silkworm infection model has the following advantages over the mouse infection model: fewer ethical issues, lower maintenance costs, less space required to keep animals, less drugs required for evaluations and shorter times for infection experiments.<sup>6,7</sup>

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the major infectious diseases and is still the greatest single infectious cause of mortality worldwide.<sup>9</sup> However, anti-TB agents in clinical use are more limited than agents available for other bacterial infections.<sup>10</sup> Therefore, new anti-TB agents with different mechanisms of action are desired. We have screened for anti-TB antibiotics using *Mycobacterium smegmatis* instead of *M. tuberculosis* because *M. smegmatis* is nonpathogenic (utilized in a normal-level laboratory), grows faster than other *Mycobacterium* spp. and is sensitive to clinically used anti-TB agents. As a result, lariatins (lasso peptides, Figure 1) and

calpinactam (a hexapeptide, Figure 1) were discovered from microorganisms in this assay, and were found to exhibit anti-TB activities *in vitro*.<sup>11–14</sup> As the next step, the *in vivo* efficacies of these microbial peptides need to be tested.

In the present study, an *in vivo*-mimic silkworm infection model with *M. smegmatis* was established and anti-mycobacterial compounds including clinically used anti-TB drugs and microbial peptides were evaluated using this silkworm infection assay.

## MATERIALS AND METHODS

### Materials

Lariatin A,<sup>11,12</sup> lysocin E<sup>1</sup> and propeptins<sup>15,16</sup> were purified from a culture broth of *R. jostii* K01-B0171, *Lysobacter* sp. RH2180-5 and *Microbispora* sp. SNA-115, respectively. Calpinactam was synthesized as reported previously.<sup>17</sup> Rifampicin (RFP), streptomycin (SM) and kanamycin (KM) were purchased from Wako Pure Chemical Industries (Osaka, Japan), isoniazid (INH) was purchased from Sigma Aldrich (St Louis, MO, USA), ethambutol (EB) was purchased from LKT Laboratories (St Paul, MN, USA) and pyrazinamide (PZA) was obtained from Tokyo Chemical Industries (Tokyo, Japan). Unless otherwise stated, all other reagents were reagent-grade commercial products. Middlebrook 7H9 broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 0.5% Tween-80 (Tokyo Chemical Industries) and 10% albumin dextrose catalase (ADC) enrichment (5% bovine serum albumin, Sigma Aldrich; 2% glucose, Wako Pure Chemical Industries; 0.85% NaCl, Wako Pure Chemical Industries) were used for the cultivation of *M. smegmatis* M341.

Fertilized silkworm eggs, *Bombyx mori* (Hu·Yo×Tukuba·Ne), were purchased from Ehime Sansyu (Ehime, Japan) and fed artificial food (Silk Mate 2S, Nihon Nosan Kogyo, Kanagawa, Japan; and Silkmate, Katakura Industries, Tokyo, Japan) until the fourth-instar larvae stage.

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We dedicate this work to Professor Satoshi Ōmura, a distinguished Nobel Prize awardee in Physiology or Medicine, 2015.

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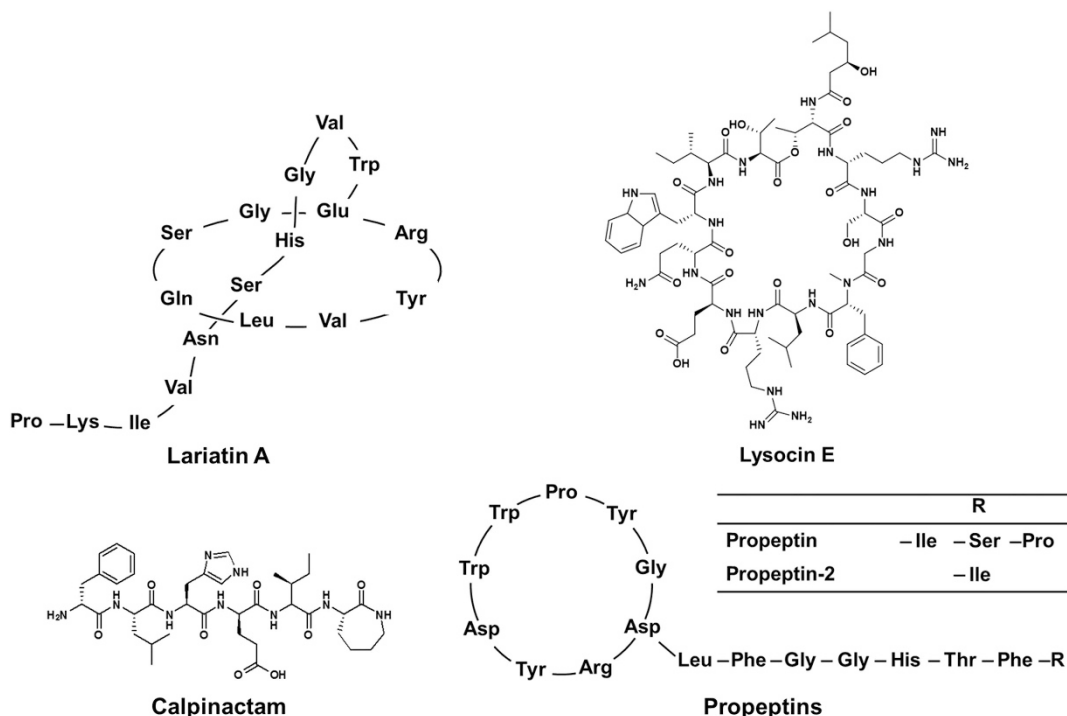


Figure 1 Structures of microbial peptides.

### Preparation of *M. smegmatis* suspension

*M. smegmatis* M341 was stored in 20% glycerol at  $-80^{\circ}\text{C}$ . The frozen stock culture was scraped with a sterile loop, inoculated in Middlebrook 7H9 broth (5 ml) in a T-25 flask (Corning, Corning, NY, USA) and cultured under static conditions at  $37^{\circ}\text{C}$  for 48 h ( $\sim 5.0 \times 10^8$  CFU  $\text{ml}^{-1}$ ).

### MIC values using the liquid microdilution method

The MIC values of the anti-TB antibiotics (INH, RFP, PZA, EB, SM and KM) and microbial peptides (lariatins A, calpinactam, lysocin E, propeptin and propeptin-2) against *M. smegmatis* were obtained using the liquid microdilution method.<sup>12</sup> An *M. smegmatis* M341 suspension was adjusted to  $1.0 \times 10^6$  CFU  $\text{ml}^{-1}$  in Middlebrook 7H9 broth containing 0.5% Tween-80 and 10% ADC enrichment. The suspension (95  $\mu\text{l}$ ) was added to each well of a 96-well microplate (Corning) with or without the test drugs (5  $\mu\text{l}$  in MeOH or water) and incubated at  $37^{\circ}\text{C}$  for 30 h. MTT reagent (5.5 mg  $\text{ml}^{-1}$  Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich; 5  $\mu\text{l}$ ) was added to each well and the cells were incubated for 16 h. After cells had been lysed with lysis buffer (40% *N,N*-dimethylformamide, Nacalai Tesque, Kyoto, Japan; 20% SDS, Wako Pure Chemical Industries; 2% acetic acid, Kanto Chemical, Tokyo, Japan; 95  $\mu\text{l}$ ), the absorbance of the lysate was measured at 570 nm using an absorption spectrometer (Power Wave X340, Bio Tek Instruments, Winooski, VT, USA). The MIC value was defined as the lowest drug concentration that showed 90% growth inhibition of *M. smegmatis*.

### Silkworm infection assay with *M. smegmatis*

Hatched silkworm larvae were raised by feeding an artificial diet containing antibiotics (Silk Mate 2S, Nihon Nosan Kogyo) in an incubator at  $27^{\circ}\text{C}$  until the fourth molting stage. On the first day of fifth-instar larvae, silkworms were fed an antibiotic-free artificial diet (Silkmate, Katakura Industries) for 24 h. On the second day, a twofold serially diluted *M. smegmatis* M341 suspension ( $0.6 \times 10^7$  to  $2.5 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$  in 50  $\mu\text{l}$  Middlebrook 7H9 broth) was injected into the hemolymph through the dorsal surface of the silkworms (2.0 g,  $n=5$ ) using a disposable 1 ml syringe with a 27-G needle (TERUMO, Tokyo, Japan). The silkworms were incubated without feed

at  $37^{\circ}\text{C}$  or  $27^{\circ}\text{C}$  unless stated otherwise, and their survival rate was measured every 2 h after drug injection.

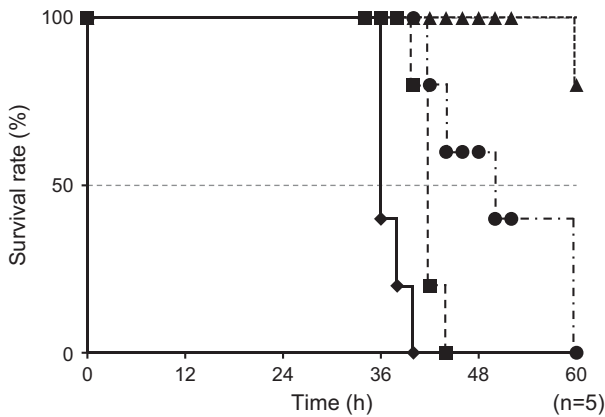
### ED<sub>50</sub> values in the silkworm infection assay with *M. smegmatis*

An *M. smegmatis* M341 suspension ( $1.25 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$  in 50  $\mu\text{l}$  Middlebrook 7H9 broth) was injected into the hemolymph of silkworm larvae (2.0 g,  $n=5$ ), followed by the injection of anti-TB-antibiotics or microbial peptides (50  $\mu\text{l}$  in water or 10% DMSO) within 30 min. Silkworms were maintained at  $37^{\circ}\text{C}$ . The survival rate of the indicated drug dose was assessed 48 h after drug injection. ED<sub>50</sub> values were defined as the amount of a drug required for a 50% survival rate, normalized per 1 g of silkworm.

## RESULTS

### Establishment of a silkworm infection model with *M. smegmatis*

*M. smegmatis* is a nonpathogenic bacterium. In order to establish a silkworm infection assay with *M. smegmatis*, the temperature ( $27^{\circ}\text{C}$  and  $37^{\circ}\text{C}$ ) for silkworm breeding and colony number of the bacterium ( $0.6 \times 10^7$  to  $2.5 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$ ) injected into silkworms were investigated. As shown in Figure 2, when silkworms injected with *M. smegmatis* ( $2.5 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$ ) were incubated at  $37^{\circ}\text{C}$ , all ( $n=5$ ) died within 42 h. In contrast, an incubation at  $27^{\circ}\text{C}$  allowed all silkworms to survive, even after 48 h. Therefore, the incubation temperature for infected silkworms was set at  $37^{\circ}\text{C}$ . Three different cell numbers ( $0.6 \times 10^7$  to  $2.5 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$ ) of *M. smegmatis* were subsequently injected into silkworms, and infected silkworms were incubated for 60 h. As shown in Figure 2, silkworms started to die after 36 to 48 h in a cell number-dependent manner. Injections of  $0.6 \times 10^7$ ,  $1.3 \times 10^7$  and  $2.5 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$  caused all silkworms to die within 60, 44 and 40 h, respectively. Therefore, the cell number of *M. smegmatis* injected into silkworms was set at  $1.25 \times 10^7$  CFU  $\text{larva}^{-1} \text{g}^{-1}$ . Furthermore, the supernatant or an autoclaved suspension of *M. smegmatis*



**Figure 2** Silkworm killing ability of *Mycobacterium smegmatis*. A suspension of the *M. smegmatis* M341 strain was diluted to the indicated cell number and injected into the silkworm hemolymph. Infected silkworms were incubated at 37 °C. The number of surviving silkworms was counted 60 h after the injection. ◆:  $2.5 \times 10^7$ ; ■:  $1.3 \times 10^7$ ; ●:  $0.6 \times 10^7$ ; and ▲: 0 CFU larva<sup>-1</sup> g<sup>-1</sup>. Experiments were performed three times and reproducible data were observed.

**Table 1** MIC and ED<sub>50</sub> values of anti-*Mycobacterium* agents against *Mycobacterium smegmatis*

Compound	MIC (μg ml <sup>-1</sup> )	ED <sub>50</sub> (μg larva <sup>-1</sup> g <sup>-1</sup> )	ED <sub>50</sub> per MIC
Isoniazid	1.56	12.5	8.0
Rifampicin	1.56	5	3.2
Pyrazinamide	>100	>50	—
Ethambutol	0.78	50	64
Streptomycin	0.78	1.5	1.9
Kanamycin	3.13	0.5	0.16
Lariat A	0.10	0.5	5
Calpinactam	0.78	5	6.4
Lysocin E	3.13	0.5	0.16
Propeptin	100	50	0.5
Propeptin-2	>100	>50	—

The MIC value was defined as the lowest antibiotic concentration that showed 90% growth inhibition of *M. smegmatis*. ED<sub>50</sub> values were defined as the amount of a drug required for 50% survival, normalized per 1 g of silkworm. Experiments were performed three times and reproducible data were observed.

showed no pathogenicity (data not shown). Based on these results, the conditions for the silkworm infection model with *M. smegmatis* were established; the bacterium injected at  $1.25 \times 10^7$  CFU larva<sup>-1</sup> g<sup>-1</sup> and infected silkworms incubated at 37 °C. Under these conditions, all silkworms die within 48 h.

#### *In vitro* anti-*M. smegmatis* activity of microbial peptides

The *in vitro* anti-*M. smegmatis* activities of clinically used anti-TB agents (INH, RFP, PZA, EB, SM and KM) and microbial peptides (lariat A, calpinactam, lysocin E, propeptin and propeptin-2, in Figure 1) were compared under the same conditions using the liquid microdilution method.<sup>12</sup> The MIC values are summarized in Table 1. INH, RFP, EB, SM and KM exhibited anti-*M. smegmatis* activities with MIC values of 1.56, 1.56, 0.78, 0.78 and 3.13 μg ml<sup>-1</sup>, respectively. However, pyrazinamide showed no anti-*M. smegmatis* activity, even at 100 μg ml<sup>-1</sup>. These results are consistent with previous findings.<sup>18</sup>

The MIC values of the five microbial peptides against *M. smegmatis* are also shown in Table 1. Lariat A, calpinactam, lysocin E and propeptin exhibited anti-*M. smegmatis* activity with MIC values of 0.10, 0.78, 3.13 and 100 μg ml<sup>-1</sup>, respectively. However, propeptin-2, an inactive form of propeptin,<sup>16</sup> showed no anti-mycobacterial activity, even at 100 μg ml<sup>-1</sup>. We confirmed the anti-*M. smegmatis* activities of lariat A, calpinactam and propeptin. Lysocin E exhibited anti-*M. smegmatis* activity for the first time. The potency of activity was in the order of lariat A > lysocin E > calpinactam >> propeptin.

#### Therapeutic efficacies of microbial peptides in the silkworm infection assay with *M. smegmatis*

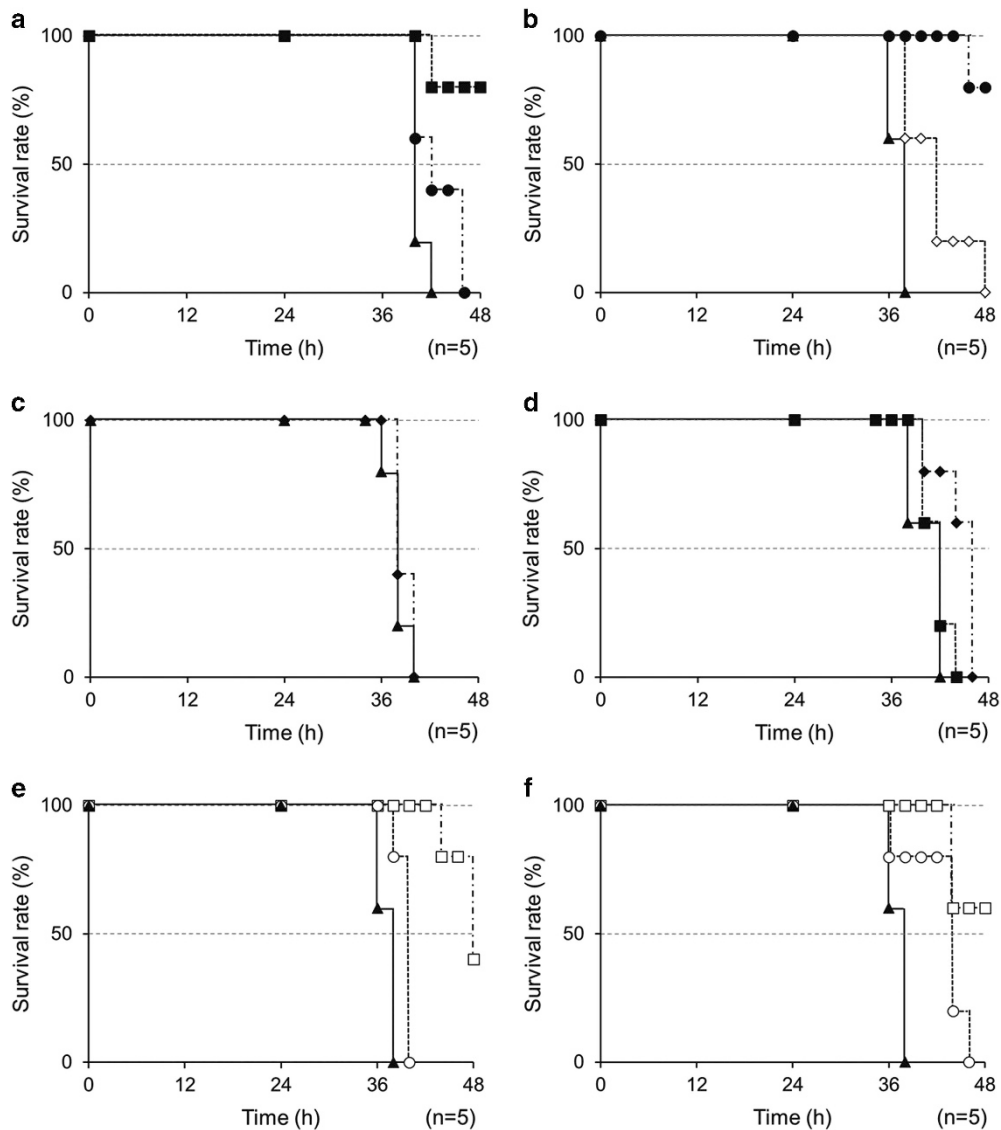
Anti-TB agents were evaluated in the silkworm infection assay ( $n = 5$ ) with *M. smegmatis*. When INH, RFP, EB, SM and KM were injected, infected silkworms survived in a dose-dependent manner (Table 1 and Figure 3). However, PZA did not exert any therapeutic effects, even at 50 μg larva<sup>-1</sup> g<sup>-1</sup> (Table 1). Furthermore, none of the anti-TB antibiotics (50 μg larva<sup>-1</sup> g<sup>-1</sup>) exhibited toxicity against silkworms, at least for 48 h (data not shown). The ED<sub>50</sub> values of anti-TB drugs against *M. smegmatis* are summarized in Table 1. Among them, the two aminoglycoside antibiotics (KM and SM) exerted strong therapeutic effects in the silkworm assay.

The five microbial peptides were evaluated in the silkworm infection assay with *M. smegmatis* ( $n = 5$ ). As shown in Figure 2, when lariat A, calpinactam, lysocin E and propeptin were injected, silkworms survived in a dose-dependent manner. The ED<sub>50</sub> values of the peptides against *M. smegmatis* are summarized in Table 1. Lariat A and lysocin E exerted strong therapeutic effects with the same ED<sub>50</sub> value of 0.5 μg larva<sup>-1</sup> g<sup>-1</sup>. Propeptin displayed very weak therapeutic effects, whereas propeptin-2 showed no effects, even at 50 μg larva<sup>-1</sup> g<sup>-1</sup> (Figure 4). None of the peptides (50 μg larva<sup>-1</sup> g<sup>-1</sup>) exhibited toxicity against silkworms, at least for 48 h (data not shown).

#### DISCUSSION

In the present study, an *in vivo*-mimic silkworm infection model with *M. smegmatis* was established. Although the incubation temperature is typically 27 °C for silkworms infected with most pathogenic microorganisms, a higher temperature of 37 °C is needed for *M. smegmatis*-infected silkworms to die within 60 h. Furthermore, a higher cell number ( $10^7$  level CFU per larva for *M. smegmatis* vs  $10^6$  level CFU per larva for other pathogenic microorganisms) is required for injection in this silkworm assay. As mycobacteria are very slow-growing bacteria, these two conditions are important for this assay to be reproducible and work well. It is important to note that the 37 °C incubation time was limited to within 60 h, even for control silkworms (Figure 2).

Clinically used anti-TB drugs were evaluated under these established conditions (Figure 3). The potency of *in vitro* anti-*M. smegmatis* activity (MIC in Table 1) was in the order of EB = SM > INH = RFP > KM >> PZA, whereas that in the silkworm assay (ED<sub>50</sub> in Table 1) was KM > SM > RFP > INH > EB >>> PZA. PZA did not exhibit any activity in the *in vitro* or silkworm assay. According to its mechanism of action on mycobacteria, PZA is converted to the active metabolite pyrazinoic acid by the enzyme pyrazinamidase in *M. tuberculosis*.<sup>19</sup> *M. smegmatis* also exhibits pyrazinamidase activity, but is resistant to PZA because PZA cannot enter the cells.<sup>19</sup> Therefore, anti-TB PZA exerts no effects on *M. smegmatis* in either assay. EB showed very potent anti-*M. smegmatis* and anti-TB activities *in vitro*; however, EB was required at the highest dose to exhibit therapeutic efficacy in *M. tuberculosis*-infected mice<sup>20</sup> and

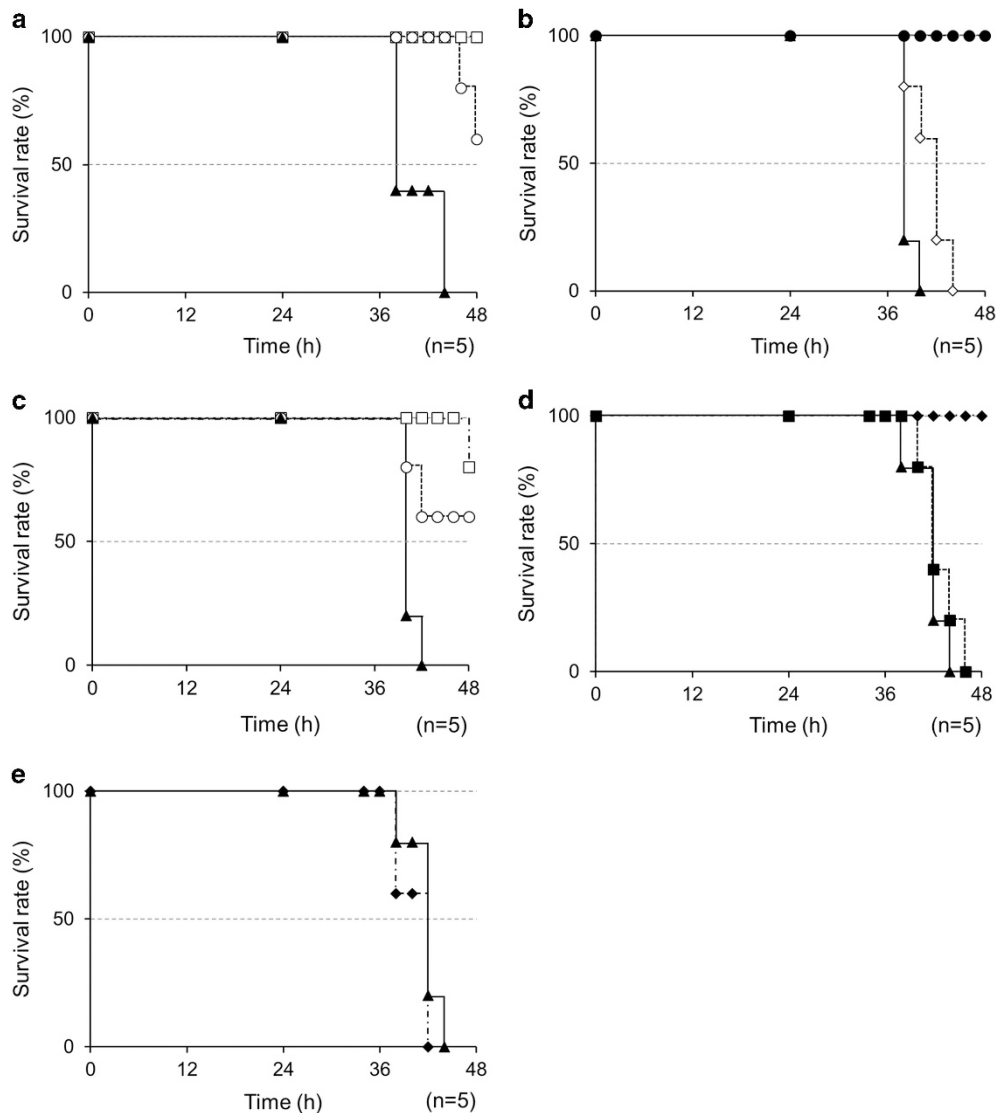


**Figure 3** Therapeutic effects of anti-tuberculosis (TB) drugs in the silkworm infection assay with *Mycobacterium smegmatis*. (a) Isoniazid (INH), (b) rifampicin (RFP), (c) pyrazinamide (PZA), (d) ethambutol (EB), (e) streptomycin (SM), and (f) kanamycin (KM). ◆: 50; ■: 25; ●: 13; ◇: 5; □: 1.5; ○: 0.5; and ▲: 0 μg larva<sup>-1</sup> g<sup>-1</sup>. Experiments were performed three times and reproducible data were observed.

*M. smegmatis*-infected silkworms. INH is a prodrug that needs to be activated by KatG, an enzyme with the dual activities of catalase and peroxidase, in order to show its anti-mycobacterial activity.<sup>21</sup> As INH displayed good efficacy in the silkworm assay, the activation reaction may occur in infected silkworms. Thus, the efficacies of the anti-TB agents in the silkworm infection assay with *M. smegmatis* were found to be consistent with those in the mouse infection assay with *M. tuberculosis*.<sup>20</sup> We consider this silkworm infection model with *M. smegmatis* to be applicable to evaluations of the *in vivo* effectiveness of candidate compounds as anti-TB agents.

Four microbial peptides reported to exhibit *in vitro* anti-mycobacterial/anti-Gram-positive bacterial activities were then evaluated in this silkworm assay. Lariatins A produced by *Rhodococcus jostii* K01-B0171 and calpinactam produced by *Mortierella alpina* FKI-4905 were discovered in the screening for antibiotics selectively active against *M. smegmatis*, and have also been reported to show *in vitro* anti-TB activities.<sup>12,13</sup> As shown in Figure 1, lariatins A forms a unique lasso

peptide,<sup>11</sup> whereas calpinactam is a hexapeptide with a caprolactam ring at its C terminus.<sup>14</sup> Lysocin E produced by *Lysobacter* sp. RH2180-5 was discovered in the *in vitro* anti-*S. aureus* assay and its therapeutic effects were confirmed in the silkworm infection assay with *S. aureus*.<sup>1</sup> Lysocin E (Figure 1) is a cyclic depsipeptide that is active against Gram-positive bacteria by targeting the bacterial membrane menaquinone. In the present study, lysocin E exhibited potent anti-*M. smegmatis* activity *in vitro* (MIC, 0.10 μg ml<sup>-1</sup>) for the first time (Table 1). Propeptin and propeptin-2 produced by *Microbispora* sp. SNA-115 were discovered in the course of screening for prolyl endopeptidase inhibitors.<sup>15,16</sup> Propeptins appear to be lasso-type peptides (Figure 1); however, direct evidence to support this has not yet been obtained. Propeptin was also reported to exhibit moderate anti-*Mycobacterium phlei* activity *in vitro*, whereas propeptin-2 lost its anti-*Mycobacterium* activity.<sup>16</sup> Corresponding *in vitro* results for the two propeptins against *M. smegmatis* were observed (Table 1). As shown in Figure 3, lariatins A, calpinactam,



**Figure 4** Therapeutic effects of microbial peptides in the silkworm infection assay with *Mycobacterium smegmatis*. (a) Lariat A, (b) calpinactam, (c) lysocin E, (d) propeptin and (e) propeptin-2. ◆: 50; ■: 25; ●: 13; ◇: 5; □: 1.5; ○: 0.5; and ▲: 0  $\mu\text{g larva}^{-1} \text{g}^{-1}$ . Experiments were performed three times and reproducible data were observed.

lysocin E and propeptin exerted therapeutic effects in a dose-dependent manner in the silkworm infection assay, whereas propeptin-2 did not, even at the highest dose. Lysocin E and lariat A were particularly potent, with the same  $\text{ED}_{50}$  values of  $0.50 \mu\text{g larva}^{-1} \text{g}^{-1}$  (Table 1). The therapeutic effects of the microbial peptides in this silkworm infection assay correlated with potencies in the *in vitro* anti-*M. smegmatis* assay (Table 1).

Hamamoto *et al.*<sup>7</sup> previously reported that the ratio of  $\text{ED}_{50}$  per MIC of a compound is an index of drug potential, and the ratio is typically  $<10$  for clinically used antibiotics. As shown in Table 1, INH, RFP, SM and KM had ratios of  $<10$ , whereas that of EB was 64. The ratios of lariat A, calpinactam, lysocin E and propeptin were  $<10$  (Table 1), indicating the potential of these peptides as anti-TB drugs.

In conclusion, we established a silkworm infection model with *M. smegmatis* as an *in vivo*-mimic evaluation for anti-TB agent development, and demonstrated the therapeutic efficacies of clinically used anti-TB drugs and microbial peptides in this infection assay.

The silkworm infection model will be utilized as a practically effective assay to develop anti-TB drugs. We identified four microbial peptides as potential anti-TB drug candidates.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### ACKNOWLEDGEMENTS

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