Original Article

Development of an *in vivo*-mimic silkworm infection model with *Mycobacterium avium* complex

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SUMMARY   *In vivo*-mimic silkworm infection models with *Mycobacterium avium* and *Mycobacterium intracellulare* were newly established to evaluate the therapeutic effects of anti-*M. avium* complex (MAC) antibiotics. Silkworms raised at 37°C died within 72 hours of an injection of *M. avium* or *M. intracellulare* (2.5×10^7 colony-forming unit (CFU)/larva·g) into the hemolymph. Clinical anti-mycobacterial (tuberculosis) antibiotics were evaluated under these conditions. Clarithromycin, kanamycin, streptomycin, amikacin, and ciprofloxacin exerted therapeutic effects in a dose-dependent manner, which was consistent with those in the mouse model. Furthermore, three effective actinomycete culture broths were selected in the screening program of our microbial broth library using the silkworm model, and four active metabolites, ohmyungsamycins A and B (**1** and **2**), chartreusin (**3**), and griseoviridin (**4**), were identified. Among these compounds, **1** showed the lowest 50% effective dose (ED_{50}) value (8.5 μg/larva·g), while **3** had the best ED_{50}/minimum inhibitory concentration (MIC) ratio (7.4). These results indicate that silkworm models are a useful tool for identifying anti-MAC antibiotics candidates with veritable therapeutic effects.

Keywords   silkworm infection model, antibiotics, *Mycobacterium avium* complex (MAC), nontuberculosis mycobacteria (NTM), natural product, microbial origin

1. Introduction

Mycobacterium avium complex (MAC) infection is mainly caused by *M. avium* and *M. intracellulare*, which are nontuberculosis mycobacteria (NTM), and is a nontuberculous mycobacterial pulmonary and intractable disorder, the incidence of which has been increasing more than that of tuberculosis in developed countries (*1,2*). Although the majority of individuals infected with MAC bacteria are asymptomatic, patients with compromised immune functions due to cancer or HIV/AIDS or those with lung disease, such as chronic obstructive pulmonary disease or cystic fibrosis, are at the highest risk of developing MAC infection. Pulmonary MAC infection progresses slowly, worsens over time, and may persist for weeks or months, and its symptoms are similar to those of tuberculosis, such as weight loss, fever, fatigue, and night sweats (*1*). Limited drugs are currently available for use in clinical practice, and the treatment approach employs the combination of first-line clarithromycin with rifampicin and ethambutol. However, this treatment is not sufficiently effective, and the emergence of bacterial resistance is a major issue because it necessitates treatment for more than one year (*3*). Although amikacin liposome inhalation suspension (ALIS) was newly approved for the treatment of MAC infection in 2018 (*4*), the development of new candidates for MAC infection with novel skeletal structures and different mechanisms of action to existing drugs is urgently needed.

In the screening of potential antibiotics against various pathogenic microorganisms from natural resources, we have conducted *in vivo*-mimic infection assays using silkworms at the early stage of drug development (*5-10*). This assay system concept is straightforward: candidate compounds or test samples are injected into pathogenic microorganism-infected silkworm larvae, and the survival rate over a few days is then monitored to assess the therapeutic effects of the sample. Furthermore, the silkworm model may be
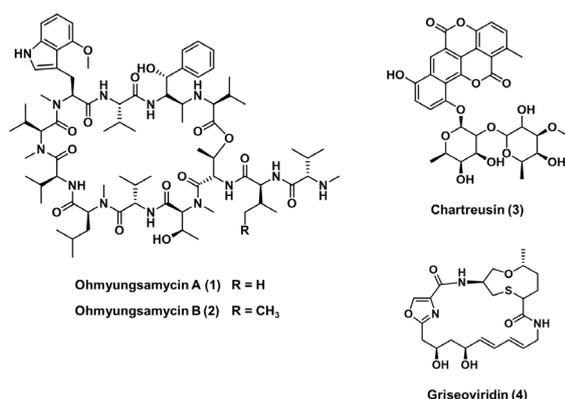
Figure 1. Structures of compounds 1-4.

evaluated more rapidly and efficiently than a mouse model. We previously established a silkworm model with *M. smegmatis* and *Mycobacteroides* (*My.*) *abscessus* for the screening anti-tuberculosis and anti-NTM agents, respectively (*11,12*), and found that lariatins (*13*), calpinactam (*14*), lysocin E (*5*), propeptin (*15*), and nosiheptide (*16*) exerted therapeutic effects in the silkworm model.

We herein successfully established a silkworm model with *M. avium* and *M. intracellulare*, which had not been achieved in previous studies, and clinical anti-tuberculosis and anti-MAC drugs were evaluated using this model. The screening study from our microbial broth library resulted in the identification of four microbial metabolites, ohmyungsamycins A (**1**) and B (**2**) (*17,18*), chartreusin (**3**) (*19*), and griseoviridin (**4**) (*20*), from the culture broths of actinomycete strains (Figure 1). We described the establishment of the silkworm model with *M. avium* and *M. intracellulare* and the *in vivo* therapeutic effects of anti-mycobacterial agents and **1-4**.

2. Materials and Methods

2.1. Materials

Kanamycin, streptomycin, amikacin, ciprofloxacin, and rifampicin were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Clarithromycin, ethambutol, isoniazid, and pyrazinamide were purchased from Tokyo Chemical Industries (Tokyo, Japan). Unless otherwise stated, all other reagents were reagent-grade commercial products. Middlebrook 7H9 broth (Becton, Dickinson and Company, NJ, USA) was blended with 0.05% Tween 80 (Tokyo Chemical Industries) and 10% ADC enrichment [5% bovine serum albumin (FUJIFILM Wako Pure Chemical Industries), 2% glucose (FUJIFILM Wako Pure Chemical Industries), and 0.85% NaCl (FUJIFILM Wako Pure Chemical Industries)] to cultivate mycobacterium strains. Seed and production media consisted of 1.0% malt extract

(Becton, Dickinson and Company), 0.4% yeast extract (Becton, Dickinson and Company), and 0.4% glucose (FUJIFILM Wako Pure Chemical Industries) to cultivate all actinomycetal strains.

2.2. Silkworms

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

2.3. Preparation of the mycobacterial suspension

M. avium JCM15430 and *M. intracellulare* JCM6384 were obtained from the Riken BioResource Research Center (Ibaraki, Japan). Both strains were stored in 20% glycerol at -80°C. Frozen stock culture (500 µL, approximately 5.0×10^8 colony-forming unit (CFU)/ mL) was inoculated into Middlebrook 7H9 broth (10 mL) in a T-25 flask (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and cultured under static conditions at 37°C for 14 days (up to approximately 1.0×10^9 CFU/mL).

2.4. Assessment of minimum inhibitory concentration (MIC) values using the liquid microdilution method

The MIC values of anti-tuberculosis antibiotics (isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, kanamycin, and clarithromycin) and four microbial metabolites (**1-4**) against *M. avium* and *M. intracellulare* were evaluated using the liquid microdilution method according to a previously established method (*11,21*). Each test strain was adjusted to 1.0×10^6 CFU/mL in Middlebrook 7H9 broth. The suspension (95 µL) was added to each well of a 96-well microplate (AS ONE, Osaka, Japan) with or without test samples (5 µL in methanol or saline) and incubated at 37°C for 5 days. Absorbance was measured at 550 nm using an absorption spectrometer (MTP-500, Corona Electric Co., Ltd., Ibaraki, Japan). The MIC value was defined as the lowest sample concentration that inhibited the growth of *M. avium* or *M. intracellulare* by 90%.

2.5. Silkworm infection model with *M. avium* and *M. intracellulare*

Hatched silkworm larvae were raised by feeding an artificial diet containing antibiotics (Silk Mate 2S) in an incubator at 27°C until the fourth molting stage. On the first day of the fifth-instar larval stage, silkworms were fed an antibiotic-free artificial diet (Silk Mate) for 24 hours. On the second day, a three-fold serially diluted *M. avium* JCM15340 or *M. intracellulare* JCM6384

suspension (0.83×10^7 to 7.5×10^7 CFU/larva·g in 50 μL saline) was injected into the hemolymph of silkworms through the dorsal surface (2.0 g, $n = 5$) using a disposable 1-mL syringe with a 27-G needle (TERUMO, Tokyo, Japan). Infected silkworms were raised without feed at 37°C unless stated otherwise, and their survival rate was measured every 3 hours after the sample injection.

2.6. Assessment of 50% effective dose (ED$_{50}$) values in the silkworm infection model with *M. avium* and *M. intracellulare*

The *M. avium* or *M. intracellulare* suspension (2.5×10^7 CFU/larva·g in 50 μL saline) was injected into the hemolymph of silkworm larvae (2.0 g, $n = 5$), followed by an injection of anti-tuberculosis antibiotics and microbial compounds (50 μL in saline or 10% dimethyl sulfoxide) within 30 minutes. Silkworms were then raised at 37ºC. The survival rate at the indicated drug dose was assessed 72 hours after its injection. ED$_{50}$ values were defined as the amount of a drug required for a 50% survival rate, normalized per 1 g of silkworm.

2.7. Isolation of **1-4**

Ohmyungsamycins A (**1**) and B (**2**): Actinomycete strain TMPU-A0334 was isolated from marine sediment collected from Tokyo Bay, Japan at a depth of 6.4 m. This strain was fermented with seed medium containing 3.64% Marine art Hi (Osaka Yakken Co., Ltd., Osaka, Japan) on a rotary shaker (180 rpm, 27°C) for 3 days, followed by a production culture on a rotary shaker (180 rpm, 27°C) for 21 days. The culture broth obtained (5.0 L) was centrifuged to separate mycelia and supernatant. After mycelia had been treated with acetone (1.0 L), the mixture was filtered and concentrated *in vacuo* to remove acetone. Aqueous solution (300 mL) was adjusted to pH 9 and extracted with an equal volume of ethyl acetate (EtOAc). The EtOAc layer was then evaporated *in vacuo* to yield a solid black material (206 mg). This material was dissolved in a small amount of methanol, applied to a Sep-Pak C18 column cartridge (5 g, Waters, MA, USA), and eluted stepwise with 0, 20, 40, 60, 80, and 100% acetonitrile in water (20 mL each). The 80% acetonitrile eluate (12.5 mg) containing **1** and **2** were purified by preparative high-performance liquid chromatography (HPLC) [Column, PEGASIL ODS SP100 (Senshu Scientific Co., Tokyo, Japan, i.d. 10 mm × 250 mm); mobile phase, 55% acetonitrile in 50 mM sodium phosphate buffer (pH 7); flow rate, 3.0 mL/min; detection, UV at 210 nm]. Under these conditions, **1** (3.1 mg, retention time (t_R) = 26 min) and **2** (3.5 mg, t_R = 34 min) were isolated as white powders.

Chartreusin (**3**): Actinomycete strain TMPU-A0405 was isolated from soil collected in Tottori prefecture, Japan. 14-day-old culture broth (1.0 L) fermented under static conditions at 27°C was extracted with acetone (600 mL) and concentrated *in vacuo* to remove acetone. The remaining aqueous solution was extracted with EtOAc (pH 3, 500 mL), and the organic layer was concentrated *in vacuo* to give a yellow solid material (259 mg). This material dissolved in a small volume of methanol was applied to a Sep-Pak C18 column cartridge (5 g) and eluted stepwise with 0, 20, 40, 60, 80, and 100% acetonitrile in H$_2$O (20 mL each). The 60% acetonitrile eluate (12.3 mg) containing **3** was evaporated, and the residue was treated with methanol (1 mL) to obtain a methanol-insoluble substance as a yellow powder of **3** (3.5 mg).

Griseoviridin (**4**): Actinomycete strain KTM7-6 was isolated from deep sea water collected from Kumejima island, Japan. 14-day-old whole culture broth fermented by the rotary shaker (180 rpm, 27°C) was extracted with acetone (600 mL), concentrated *in vacuo* to remove acetone, and extracted with EtOAc (200 mL) to obtain the EtOAc extract (86.6 mg). The extract dissolved in methanol was subjected to a Sep-Pak C18 column cartridge (5 g) and eluted stepwise with 0, 20, 40, 60, 80, and 100% acetonitrile in water (20 mL each). The 60% acetonitrile eluate was evaporated *in vacuo*, and **4** was isolated as a white powder (3.5 mg).

The structures of **1-4** were identified by comparing their various spectroscopic data, including nuclear magnetic resonance (NMR) and mass spectrometry (MS) experiments, with those described in the literature (*17-20*) as ohmyungsamycin A (**1**), ohmyungsamycin B (**2**), chartreusin (**3**), and griseoviridin (**4**), respectively (Figure 1).

3. Results

3.1. Establishment of the silkworm infection model with *M. avium* and *M. intracellulare*

To establish the silkworm model with *M. avium* and *M. intracellulare*, the cell concentrations of the mycobacterium injected into silkworms were examined. Silkworms were infected with three different concentrations (0.83×10^7, 2.5×10^7, and 7.5×10^7 CFU/larva·g) and observed for 96 hours under the incubation at 37ºC. Silkworms infected with *M. avium* and *M. intracellulare* died in a cell concentration-dependent manner. All silkworms infected with *M. avium* at 0.83×10^7, 2.5×10^7, and 7.5×10^7 CFU/larva·g died within 81, 72, and 64 hours, respectively (Figure 2A). The infection with *M. intracellulare* at 0.83×10^7, 2.5×10^7, and 7.5×10^7 CFU/larva·g caused death within 81, 68, and 60 hours, respectively (Figure 2B). Therefore, the cell concentrations of *M. avium* and *M. intracellulare* were fixed at 2.5×10^7 CFU/larva·g. The supernatant or autoclaved suspensions of *M. avium* and *M. intracellulare* had no pathogenicity against silkworms (data not shown). Based on these results, the

silkworm model with *M. avium* and *M. intracellulare* was established; these mycobacteria were injected into the silkworm hemolymph at 2.5×10^7 CFU/larva·g, and infected silkworms were then raised at 37°C. Under these conditions, all silkworms died within 72 hours.

3.2. *In vitro* anti-*M. avium* and *M. intracellulare* activities of anti-tuberculosis antibiotics

The *in vitro* anti-*M. avium* and *M. intracellulare* activities of clinically used anti-tuberculosis drugs (clarithromycin, kanamycin, streptomycin, amikacin, ciprofloxacin, rifampicin, ethambutol, isoniazid, and pyrazinamide) were compared using the liquid microdilution method. MIC values are summarized in Table 1. Clarithromycin, kanamycin, streptomycin, amikacin, ciprofloxacin, rifampicin, ethambutol, and isoniazid inhibited the growth of *M. avium* with MIC values of 0.098, 3.1, 1.6, 1.6, 0.20, 0.20, 13, and 1.6 µg/mL, respectively. The anti-*M. intracellulare* activities of clarithromycin, kanamycin, streptomycin, amikacin, ciprofloxacin, rifampicin, ethambutol, and isoniazid showed MIC values of 0.012, 1.6, 0.78, 1.6, 0.39, 0.049, 1.6, and 3.1 µg/mL, respectively. However, pyrazinamide did not exhibit anti-*M. avium* or *M. intracellulare* activity, even at 50 µg/mL.

3.3. Therapeutic effects of anti-tuberculosis antibiotics in the silkworm infection model with *M. avium* and *M. intracellulare*

Anti-tuberculosis antibiotics were then assessed in the established silkworm model with *M. avium* and *M. intracellulare* ($n = 5$), and their ED_{50} values are listed in Table 1. Treatments with clarithromycin, kanamycin, streptomycin, amikacin, and ciprofloxacin exerted therapeutic effects in the silkworm model with *M. avium* in a dose-dependent manner with ED_{50} values of 23, 23, 20, 140, and 140 µg/larva·g, respectively (Table 1, Figure 3). Similarly, clarithromycin, kanamycin, streptomycin, and amikacin exerted therapeutic effects in the silkworm model with *M. intracellulare* with ED_{50} values of 42, 27, 84, and 160 µg/larva·g (Table 1, Figure 4). However, rifampicin, ethambutol, isoniazid, and pyrazinamide did not exert therapeutic effects, even at 200 µg/larva·g (Table 1, Figures 3 and 4). Moreover, none of the anti-tuberculosis antibiotics (200 µg/larva·g)
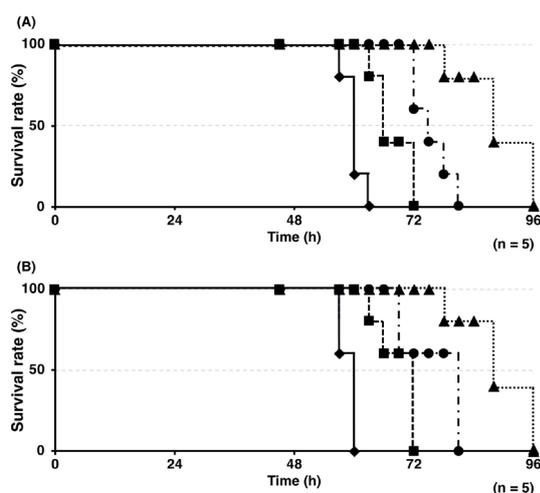
Figure 2. Silkworm-killing abilities of (A) *M. avium* and (B) *M. intracellulare*. Suspensions of the *M. avium* JCM15340 and *M. intracellulare* JCM6384 strains were diluted to the indicated cell number and injected into the silkworm hemolymph. Infected silkworms were incubated at 37°C. The number of surviving silkworms was counted 96 h after the injection. ♦: 7.5×10^7, ■: 2.5×10^7, ●: 0.83×10^7, ▲: 0 CFU/larva·g, Experiments were performed three times and reproducible data were observed.

Table 1. Anti-microbial properties of anti-mycobacterium agents and compounds 1-4 against *M. avium* and *M. intracellulare*

	Mycobacterium avium			*Mycobacterium intracellulare*		
	MIC (µg/mL)	ED_{50} (µg/larva·g)	ED_{50}/MIC	MIC (µg/mL)	ED_{50} (µg/larva·g)	ED_{50}/MIC
Clarithromycin	0.098	23	230	0.012	42	3500
Kanamycin	3.1	23	7.4	1.6	27	17
Streptomycin	1.6	20	13	0.78	84	110
Amikacin	1.6	140	88	1.6	160	100
Ciprofloxacin	0.20	140	700	0.39	> 200	–
Rifampicin	0.20	> 200	–	0.049	> 200	–
Ethambutol	13	> 200	–	1.6	> 200	–
Isoniazid	1.6	> 200	–	3.1	> 200	–
pyrazinamide	> 50	> 200	–	> 50	> 200	–
Ohmyungsamycin A (**1**)	0.39	8.5	22	0.20	40	200
Ohmyungsamycin B (**2**)	1.6	42	26	1.6	> 50	–
Chartreusin (**3**)	3.1	23	7.4	3.1	> 50	–
Griseoviridin (**4**)	1.6	35	22	0.78	> 50	–

The MIC value was defined as the lowest compound concentration that inhibited the growth of *M. avium* and *M. intracellulare* by 90%. The ED_{50} value was defined as the amount of the compound required for 50% survival, normalized per 1 g of silkworm. Experiments were performed three times and reproducible data were observed.
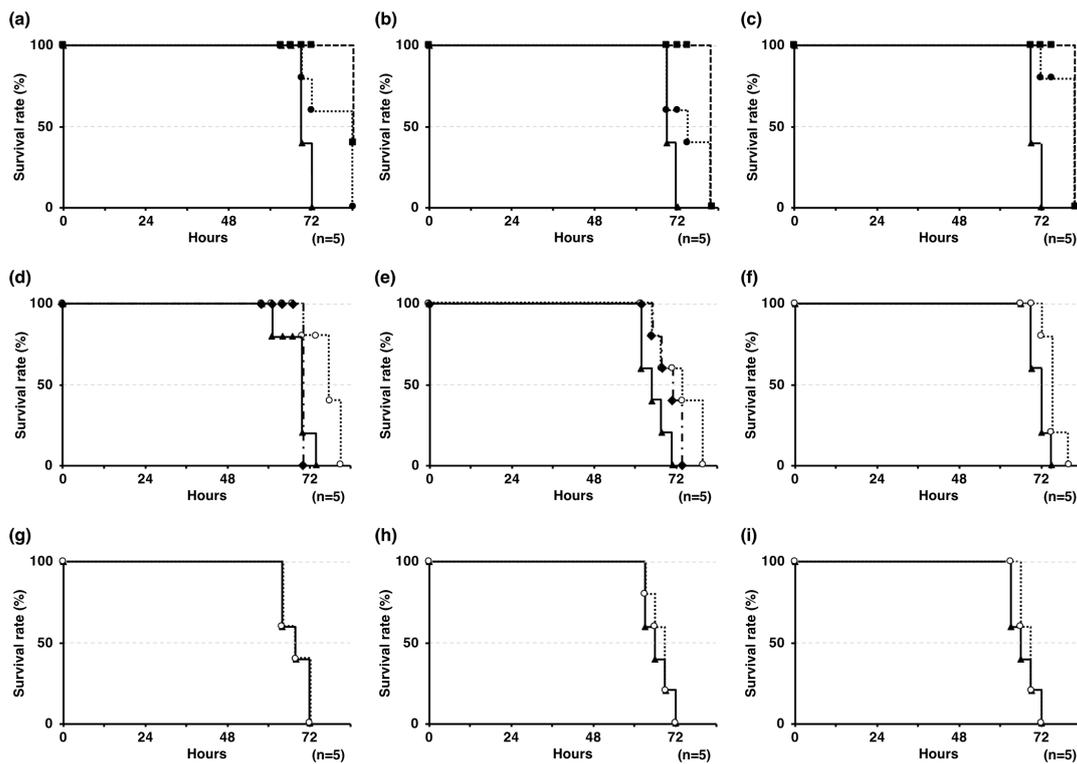
Figure 3. Therapeutic effects of anti-mycobacterium drugs in the silkworm infection assay with *M. avium*. (a) Clarithromycin (CAM), **(b)** kanamycin (KM), **(c)** streptomycin (SM), **(d)** amikacin (AMK), **(e)** ciprofloxacin (CPFX), **(f)** rifampicin (RFP), **(g)** ethambutol (EB), **(h)** isoniazid (INH), and **(i)** pyrazinamide (PZA). ○: 200, ♦: 100, ■: 50, ●: 25, ▲: 0 μg/larva·g. Experiments were performed three times and reproducible data were observed.
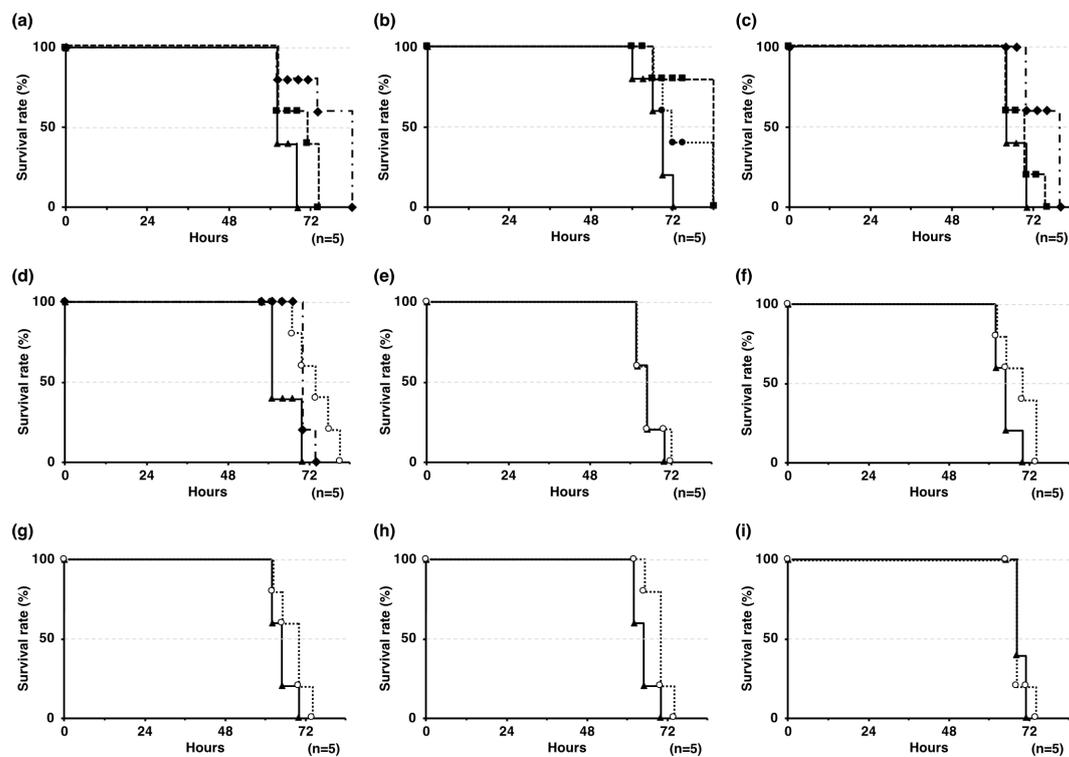
Figure 4. Therapeutic effects of anti-mycobacterium drugs in the silkworm infection assay with *M. intracellulare*. (a) Clarithromycin (CAM), **(b)** kanamycin (KM), **(c)** streptomycin (SM), **(d)** amikacin (AMK), **(e)** ciprofloxacin (CPFX), **(f)** rifampicin (RFP), **(g)** ethambutol (EB), **(h)** isoniazid (INH), and **(i)** pyrazinamide (PZA). ○: 200, ♦: 100, ■: 50, ●: 25, ▲: 0 μg/larva·g. Experiments were performed three times and reproducible data were observed.

exhibited toxicity against silkworms, at least for 72 hours (data not shown).

3.4. Screening of anti-*M. avium* and *M. intracellulare* compounds in the silkworm infection model

We started screening for new types of anti-*M. avium* and *M. intracellulare* compounds using the established silkworm model; approximately 1,500 microbial broths of terrestrial and marine fungi and actinomycetes were evaluated. Consequently, three culture broths of actinomycete strains TMPU-A0334, TMPU-A0405, and KTM7-6 exerted therapeutic effects in the silkworm model. The bioactivity-guided separation of the broths led to the isolation of four microbial metabolites, ohmyungsamycins A (**1**) and B (**2**) from strain TMPU-A0334, chartreusin (**3**) from strain TMPU-A0405, and griseoviridin (**4**) from strain KTM7-6. Compounds **1** and **2** were initially reported as cyclic peptides produced by the marine-derived *Streptomyces* sp. in 2013 (*17*), and the structure of **2** was revised by a total synthesis study in 2018 (*18*). Compound **3** was discovered in various actinomycetal culture broths following its first isolation from *Streptomyces* sp. in 1955 (*19*). Furthermore, **4** was initially isolated from *Streptomyces* sp. in 1956 (*20*), and we recently rediscovered its *in vitro* growth inhibitory activity against *M. avium* and *M. intracellulare* (*21*).

3.5. *In vitro* anti-*M. avium* and *M. intracellulare* activities of **1**-**4**

The *in vitro* anti-*M. avium* and *M. intracellulare* activities of **1**-**4** were measured using the liquid microdilution method. Compound **1** showed the most potent *in vitro* anti-*M. avium* activity with a MIC value of 0.39 g/mL, while **2**-**4** had MIC values of 1.6, 3.1, and 1.6 g/mL, respectively (Table 1). The MIC values of **1**-**4** against *M. intracellulare* were equivalent to those against *M. avium*.

3.6. Therapeutic effects of **1**-**4** in the silkworm infection model with *M. avium* and *M. intracellulare*

Compounds **1**-**4** were evaluated in the silkworm model with *M. avium* and *M. intracellulare* ($n = 5$), and their ED_{50} values are summarized in Table 1. As shown in Figure 5, when **1**-**4** were administered to silkworms infected with *M. avium*, moderate therapeutic effects were confirmed in a dose-dependent manner with ED_{50} values of 8.5, 42, 23, and 35 g/larva·g, respectively. However, in the silkworm model with *M. intracellulare*, only silkworms treated with **1** survived (ED_{50}; 40 g/larva·g), and **2**-**4** showed no effects in the *M. intracellulare* infection model, even at 50 g/larva·g (Figure 6). Compounds **1**-**4** did not exhibit any toxicity toward silkworms at least for 72 hours (data not shown).

The *in vitro* and *in vivo* experimental values of **1**-**4**

were used to calculate ED_{50}/MIC ratios, an index of the drug potential of antibiotics (*22*), which were listed in Table 1. Among them, **3** showed the best ED_{50}/MIC rate (7.4) in the silkworm model with *M. avium*, while **1**, **2**, and **4** showed similar rates (22 to 26). In contrast, only **1** exhibited a high ED_{50}/MIC rate (200) in the silkworm model with *M. intracellulare*.

4. Discussion

We herein successfully established an *in vivo*-mimic silkworm infection model with *M. avium* and *M. intracellulare*, which are slowly growing mycobacteria. We previously reported that silkworms infected with *M. smegmatis* (1.3×10^7 CFU/larva·g) and *My. abscessus* (3.8×10^7 CFU/larva·g), which are rapidly growing mycobacteria, died within 48 hours of infection (*11,12*). Based on these findings, further investigations showed that silkworms needed a longer time (for 72 hours) and a higher cell concentration (2.5×10^7 CFU/larva·g) for death due to infection with *M. avium* and *M. intracellulare*.

Clarithromycin is currently the standard therapy for MAC disease as the first-line drug in clinical practice. Combination therapy with rifampicin and ethambutol is simultaneously used to prevent the emergence of *M. avium* and *M. intracellulare* resistant to clarithromycin. The intravenous administration of aminoglycosides (streptomycin, kanamycin, or amikacin) twice or thrice weekly for at least two months is recommended in severe cases. The *in vitro* and *in vivo* anti-mycobacterial activities of various anti-tuberculosis drugs against MAC bacteria have been reported, as described below. The majority of these drugs were found to be active *in vitro*, except for pyrazinoic acid (*23*), the active metabolite converted from pyrazinamide. Among them, only clarithromycin (50-200 mg/kg) and aminoglycosides (kanamycin; 20 mg/kg, streptomycin; 150 mg/kg, and amikacin; 100-220 mg/kg) exerted therapeutic effects as a single agent in *in vivo* mouse infection models with MAC bacteria. On the other hand, rifampicin (10 mg/kg), isoniazid (40 mg/kg), ethambutol (20-100 mg/kg), and ciprofloxacin (40 mg/kg) had marginal effects (*24-28*). However, high doses of fluoroquinolones (moxifloxacin; 100 mg/kg and levofloxacin; 200 mg/kg) exerted therapeutic effects in MAC-infected mice, suggesting that ciprofloxacin also needs to be administered at higher doses to achieve greater therapeutic effects (*29*). These findings are consistent with the present results obtained using the silkworm model with *M. avium*. In comparisons, the therapeutic effects of these drugs were weaker in the silkworm model with *M. intracellulare* than in the model with *M. avium*. *M. intracellulare* has been reported to be more pathogenic and refractory than *M. avium* in clinical practice (*30,31*), which is consistent with the results obtained in the silkworm model.
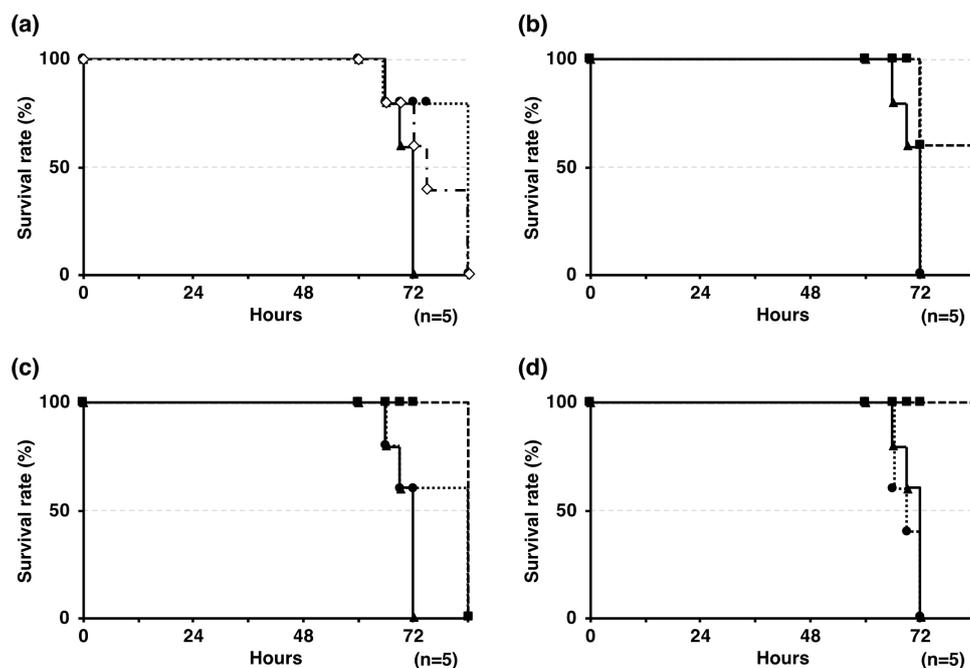
Figure 5. Therapeutic effects of compounds 1-4 in the silkworm infection assay with *M. avium*. (a) Ohmyungsamycin A (**1**), **(b)** ohmyungsamycin B (**2**), **(c)** chartreusin (**3**), and **(d)** griseoviridin (**4**). ■: 50, ●: 25, ◊: 13, ▲: 0 μg/larva·g. Experiments were performed three times and reproducible data were observed.
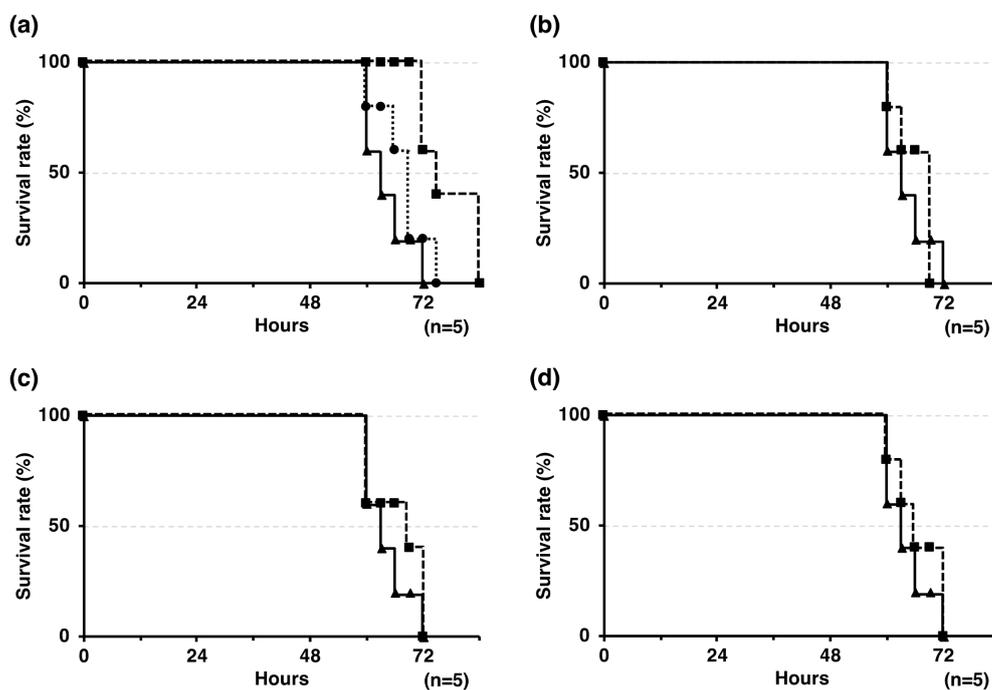
Figure 6. Therapeutic effects of compounds 1-4 in the silkworm infection assay with *M. intracellulare*. (a) Ohmyungsamycin A (**1**), **(b)** ohmyungsamycin B (**2**), **(c)** chartreusin (**3**), and **(d)** griseoviridin (**4**). ■: 50, ●: 25, ▲: 0 μg/larva·g. Experiments were performed three times and reproducible data were observed.

Accordingly, we concluded that the silkworm model may be used to evaluate the *in vivo* effects of anti-MAC drug candidates.

Hamamoto and co-workers previously reported that the ED_{50}/MIC ratio, indicating an index of drug potential, of common clinical antibiotics was less than 10 (*22*). In the present study, the ratio of kanamycin had the best score of 7.4 in the silkworm model with *M. avium*, while those of clarithromycin, streptomycin, amikacin, and ciprofloxacin were higher than 10, suggesting that the efficiencies of the majority of the anti-tuberculosis drugs tested were poor in the silkworm

model with *M. avium*.

We then screened our microbial broth library using the silkworm model, and identified four potential compounds, ohmyungsamycins A and B (**1** and **2**), chartreusin (**3**), and griseoviridin (**4**), from the actinomycete strains TMPU-A0334, TMPU-A0405, and KTM7-6, respectively. Compounds **1** to **4** exerted therapeutic effects in a dose-dependent manner in the silkworm model with *M. avium*. Of these, **1** exerted potent therapeutic effect with the lowest ED_{50} value of 8.5 µg/larva·g in all tested compounds, including anti-tuberculosis drugs. Moreover, the ED_{50}/MIC ratios of **1**, **2**, and **4** were relatively high (ratio; 22 to 26), while that of **3** only was < 10 (ratio; 7.4), similar to that of KM. Therefore, the therapeutic effects of **1** and **3** need to be examined in a mouse model.

We previously discovered a unique lasso peptide lariatin A, produced by *Rhodococcus jostii* K01-B0171, in the screening of anti-tuberculosis antibiotics using *M. smegmatis* (*13*). Lariatin A also exerted therapeutic effects in the silkworm model with *M. smegmatis* (ED_{50}; 0.5 µg/larva·g) and *My. abscessus* (ED_{50}; 4.4 µg/larva·g) (*11,12*). In the present study, lariatin A exhibited anti-*M. avium* and anti-*M. intracellulare* activities with MIC values of 1.56 and 1.56 µg/mL, respectively, in the microdilution assay (data not shown), but no therapeutic effects in the silkworm model with *M. avium* and *M. intracellulare* (ED_{50}; > 50 µg/larva·g). Interestingly, the therapeutic effects of **1**, which were potent in the silkworm model with *M. avium* and *M. intracellulare*, were negligible in the silkworm model with *M. smegmatis* and *My. abscessus* (*12*). Many pathogenic mycobacteria are generally slow-growing. Therefore, the evaluation of test compounds by silkworm models using mycobacteria with different growth rates is critical for obtaining a more detailed understanding of actual medicinal effects that cannot be distinguished by *in vitro* activity.

In summary, we herein established silkworm models with *M. avium* and *M. intracellulare* to screen and develop new anti-MAC drugs. The reliability of the silkworm model was supported by comparisons of the therapeutic effects of clinically used anti-mycobacterium drugs between the silkworm and mouse models. The evaluation period of test compounds in the silkworm model was successfully reduced from 4 weeks in the mouse model to 4 days in the silkworm model. Furthermore, we identified four compounds as potential anti-MAC candidates from our microbial broth collection within a short period. Thus, the silkworm model has potential as a practical *in vivo*-mimic model for discovering a new class of anti-MAC drugs with therapeutic effects.

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