scientific reports

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Pharmacokinetics of anti-*Mycobacterium avium-intracellulare* disease drugs in silkworms

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Pulmonary *Mycobacterium avium-intracellulare* complex (MAC) disease is a typical non-tuberculous mycobacterial infection. The incidence of pulmonary MAC is increasing worldwide. This study aimed to clarify the pharmacokinetic parameters of anti-pulmonary MAC disease drugs in silkworms. The pharmacokinetic parameters investigated included maximum concentration, area under the concentration–time curve, total clearance, and volume of distribution at steady-state. In addition, protein-binding rates, fat body transferability, and drug-drug interactions were examined. Antibiotic concentrations were measured using a validated high-performance liquid chromatography-mass spectrometry method. Among the antibiotics investigated, amikacin was not eliminated from silkworms during the 48-h observation period. In contrast, dose-proportional pharmacokinetics were observed in silkworms for all antibiotics tested, except for amikacin. Protein-binding rates in hemolymph for clarithromycin, azithromycin, rifampicin, ethambutol, and amikacin were 39.6 ± 3.0%, 39.5 ± 4.3%, 76.3 ± 3.2%, 20.9 ± 4.2%, and 73.1 ± 4.7%, respectively (mean ± standard deviation). The distribution of antibiotics in the fat bodies of silkworms was related to drug lipophilicity. No drug-drug interactions were observed in the silkworms. The pharmacokinetics of these drugs in silkworms differed significantly from those in humans. Therefore, while it is challenging to predict the pharmacokinetics of these drugs in humans based on silkworm data, the silkworm infection model has facilitated a comprehensive assessment of the relationship between antibiotic exposure and efficacy.

The incidence and prevalence of pulmonary *Mycobacterium avium* and *M. intracellulare* (*M. avium-intracellulare* complex; MAC) disease are increasing worldwide[1,2]. Although long-term multidrug combination therapy is recommended to treat this disease, the culture conversion rate has been reported to be 55–65%[3]. In addition, there are concerns about deviations from standard treatment owing to adverse events, leading to the high emergence of macrolide resistance[4]. Therefore, practicing personalized medicine that maximizes beneficial effects and minimizes side effects is important in the treatment of pulmonary MAC disease. However, the relationship between drug exposure and antimicrobial effects, which is necessary information for practicing personalized medicine, has not been clarified owing to the difficulty in evaluating multidrug combination therapy.

Although macrolides have promising effects in the treatment of pulmonary MAC disease, macrolide monotherapy is not recommended because of the high emergence of macrolide resistance[1]. The combined use of antibiotics, such as ethambutol, is aimed at preventing the development of macrolide resistance[5]. Thus, rather than examining the antibacterial effect independently, doing so while preventing macrolide resistance under antibiotic combination therapy with macrolides is important.

In general, mammals are used to evaluate drug exposure and response relationships. Nevertheless, the costs and ethical issues are too high to examine all combinations of multidrug combination therapies in mammals. Therefore, we focused on a recently developed method using silkworm-MAC infection models[6]. Silkworms have various advantages as experimental animals, such as low rearing costs and fewer ethical concerns[7]. Moreover,

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silkworms have been used as models of bacterial and *M. abscessus* (a typical acid-fast bacterium) infections to evaluate antimicrobial efficacy and bacterial virulence[8,9]. Consequently, silkworm infection models are considered suitable for investigating the association between antimicrobial exposure and efficacy under concomitant antimicrobial conditions.

To calculate the exposure of antibiotics in silkworms, it is necessary to determine the pharmacokinetic parameters of antibiotics that link drug dosage and exposure relationships. Among anti-pulmonary MAC disease drugs, only the pharmacokinetics of rifampicin have been reported in silkworms[10]. Accordingly, this study aimed to investigate the pharmacokinetics of anti-pulmonary MAC disease drugs to determine the relationship between dosage and exposure in silkworms. This is a preliminary investigation, not intended for human pharmacokinetic extrapolation, but to explore the correlation between exposure and efficacy using a silkworm infection model.

Results

Single-dose study

The anti-pulmonary MAC disease drugs, clarithromycin, azithromycin, rifampicin, ethambutol, clofazimine, and amikacin, were individually injected into 5th instar-day 1 and -day 5 silkworms. Weights of the 5th instar-day 1 and -day 5 silkworms were approximately 2.0 g and 5.0 g, respectively. The drug concentration–time curves are shown in Fig. 1, and the injected antibiotic doses and estimated pharmacokinetic parameters shown in Table 1.

All the measured concentrations were above the lower limit of quantification. The pharmacokinetic parameters of each antibiotic were similar between the 5th instar-day 1 and -day 5 silkworms, except for amikacin. Amikacin was barely eliminated from silkworms, and the estimated total clearance and area under the concentration–time curves significantly different between the 5th instar-day 1 and -day 5 silkworms. The estimated rifampicin total clearance and volume of distribution at steady-state were similar to those previously reported (Table 1).

Dose proportionality study

Three different doses of antibiotics were injected into the silkworms to confirm dose proportionality. The pharmacokinetic parameters of the maximum concentration and area under the concentration–time curves are shown in Table 2. Exposure to antibiotics increased in a dose-dependent manner, except for amikacin. Amikacin exposure was highest at intermediate doses, and no dose proportionality was observed. For clofazimine, dose proportionality was observed only for the area under the concentration–time curve. In contrast, the maximum concentration of clofazimine remained almost constant even when the dose increased.

Protein-binding rates of antibiotics in silkworm hemolymph

Protein-binding rates other than those for clofazimine were measured (Table 3). The protein-binding rates in silkworm hemolymph for clarithromycin, azithromycin, rifampicin, ethambutol, and amikacin were 39.6 ± 3.0%,

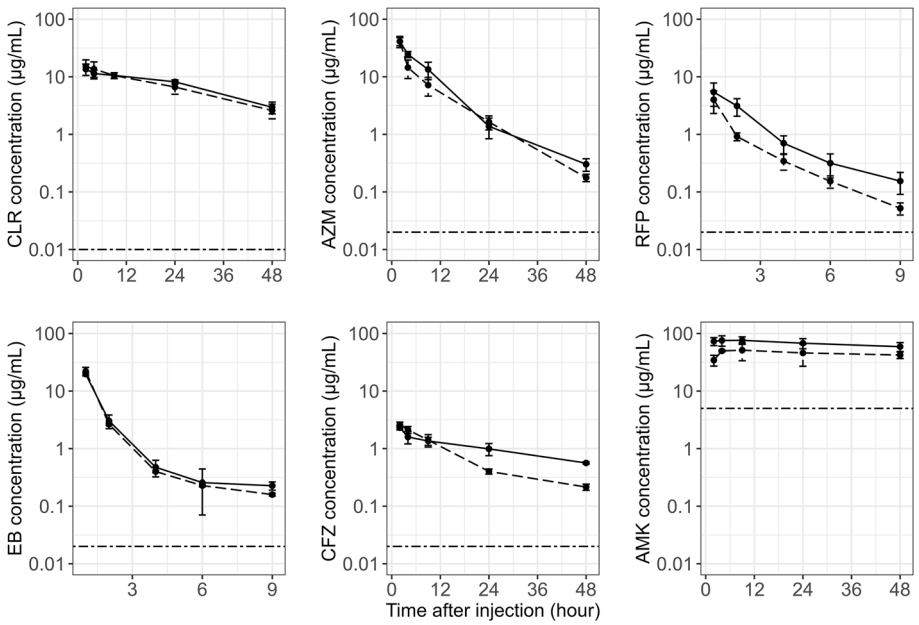

Figure 1. Antibiotic concentration–time curves. The Y-axis displays the logarithmic concentration of each drug and the X-axis the time elapsed after drug injection. Error bars represent the standard deviation. Dashed and solid lines depict the drug concentration–time curves for the 5th instar silkworms on the first and fifth days. Dotted-dashed lines indicate the lower limits of quantification. CLR, clarithromycin; AZM, azithromycin; RFP, rifampicin; EB, ethambutol; CFZ, clofazimine; AMK, amikacin.

Drug	Silkworm state	Dose (mg/kg)	Cmax (μg/mL)	AUC (μg hour/mL)	Half-life (hour)	CL (mL/min/kg)	*Vss* (mL/kg)
Clarithromycin	5th instar-day 1	10	16.1 ± 4.7	437.5 ± 60.6	19.4 ± 4.0	0.8 ± 0.1	1244.3 ± 373.5
	5th instar-day 5	10	13.6 ± 3.0	477.5 ± 62.9	21.2 ± 5.2	0.7 ± 0.1	1242.7 ± 211.6
Azithromycin	5th instar-day 1	10	41.2 ± 6.6	362.8 ± 67.8	7.5 ± 0.8	0.5 ± 0.1	154.5 ± 27.9
	5th instar-day 5	10	41.2 ± 9.1	410.6 ± 37.8	7.5 ± 0.5	0.4 ± 0.04	159.8 ± 30.0
Rifampicin	5th instar-day 1	20	4.0 ± 1.7	16.0 ± 8.9	1.9 ± 0.4	24.8 ± 10.6	1573.5 ± 916.0
	5th instar-day 5	20	5.4 ± 2.4	18.0 ± 7.0	2.6 ± 1.0	21.1 ± 10.1	2791.3 ± 1959.0
	Reference[10]	25			1.0	35.2	3100.0
Ethambutol	5th instar-day 1	20	22.0 ± 3.8	123.0 ± 38.9	3.9 ± 0.4	2.9 ± 0.9	79.6 ± 39.4
	5th instar-day 5	20	19.7 ± 1.2	94.1 ± 9.4	6.7 ± 4.2	3.6 ± 0.3	176.2 ± 48.8
Clofazimine	5th instar-day 1	2	2.6 ± 0.3	49.7 ± 1.6	29.2 ± 11.5	0.7 ± 0.02	1122.1 ± 513.0
	5th instar-day 5	2	2.3 ± 0.2	81.9 ± 7.7	35.1 ± 14.9	0.4 ± 0.04	1130.9 ± 298.5
Amikacin	5th instar-day 1	20	58.1 ± 10.5	7687.2 ± 2102.7	92.6 ± 48.6	0.05 ± 0.01	366.2 ± 120.8
	5th instar-day 5	20	77.7 ± 13.9	13,781.1 ± 5949.0	120.5 ± 56.5	0.03 ± 0.01	255.3 ± 40.8

Table 1. Estimated pharmacokinetic parameters. Data are shown as the mean ± standard deviation. *Cmax* maximum concentration, *AUC* area under the concentration–time curve, *CL* total clearance, *Vss* volume of distribution at steady-state.

Antibiotics	Dose	Cmax (μg/mL)	AUC (μg·hour/mL)
Clarithromycin	5 mg/kg	7.6 ± 0.4	269.4 ± 47.0
	10 mg/kg	13.6 ± 5.9	457.2 ± 55.3
	20 mg/kg	26.2 ± 3.2	1265.1 ± 327.4
Azithromycin	5 mg/kg	16.7 ± 1.4	163.4 ± 8.1
	10 mg/kg	41.2 ± 39.9	410.6 ± 37.8
	20 mg/kg	54.0 ± 4.6	714.3 ± 100.7
Rifampicin	10 mg/kg	1.6 ± 0.1	4.7 ± 0.3
	20 mg/kg	4.6 ± 0.9	11.9 ± 0.5
	40 mg/kg	9.6 ± 2.2	23.4 ± 0.3
Ethambutol	10 mg/kg	8.3 ± 2.1	32.8 ± 11.4
	20 mg/kg	19.7 ± 1.2	94.1 ± 9.4
	40 mg/kg	50.7 ± 5.8	155.8 ± 8.0
Clofazimine	2 mg/kg	2.3 ± 0.2	79.6 ± 6.9
	4 mg/kg	2.3 ± 0.6	255.6 ± 47.9
	10 mg/kg	3.0 ± 0.3	448.1 ± 122.3
Amikacin	5 mg/kg	20.5 ± 1.7	3535.3 ± 597.2
	10 mg/kg	55.2 ± 2.6	24,647.5 ± 15,637.0
	20 mg/kg	77.7 ± 13.9	13,781.1 ± 5949.0

Table 2. Antibiotics exposure for three doses. Data are shown as the mean ± standard deviation. *Cmax* maximum concentration, *AUC* area under the concentration–time curve.

Antibiotics	In hemolymph (current study)	In human serum or plasma[references]
Clarithromycin	39.6 ± 3.0%	40–72%[11]
Azithromycin	39.5 ± 4.3%	12–50%[12]
Rifampicin	76.3 ± 3.2%	72–91%[13]
Ethambutol	20.9 ± 4.2%	20–30%[13]
Amikacin	73.1 ± 4.7%	0%[14]

Table 3. Protein-binding rates. Data are shown as the mean ± standard deviation.

39.5 ± 4.3%, 76.3 ± 3.2%, 20.9 ± 4.2%, and 73.1 ± 4.7%, respectively (mean ± standard deviation). Although the protein-binding rate of amikacin in silkworms was significantly higher than that measured in humans, the other antibiotics showed comparable protein-binding rates[11–14] (Table 3).

Fat body transferability

Antibiotic distribution characteristics in the fat bodies of silkworms were calculated by dividing the concentration in the fat bodies with that in the hemolymph (Table 4). The concentration ratio could not be calculated for rifampicin and ethambutol 24 h after injection because the hemolymph concentration was below the lower limit of quantification. Distribution characteristics were associated with logP values obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

Drug-drug interactions

The effect of concomitant antibiotic use on antibiotic exposure was also investigated (Table 5). Amikacin was excluded from this study because it was barely eliminated from the silkworms and its exposure could thus not be accurately estimated. The antibiotics examined in this study showed constant exposure, regardless of whether they were used in combination with other antibiotics. Therefore, no drug-drug interactions between the anti-pulmonary MAC disease drugs were confirmed in the 5th instar silkworms.

Discussion

To the best of our knowledge, this is the first study to investigate the pharmacokinetics of anti-pulmonary MAC disease drugs in silkworms.

Hamamoto et al. reported that the pharmacokinetics of antimicrobial agents, such as chloramphenicol, tetracycline, vancomycin, rifampicin, micafungin, and fluconazole, are fundamentally similar between silkworms and mammals[10]. In this study, the pharmacokinetics of clarithromycin, azithromycin, ethambutol, clofazimine, and amikacin in silkworms are examined. However, many of the drugs showed significant differences in pharmacokinetics compared to humans. The half-lives of clarithromycin and azithromycin in humans are 5.9 and 65 h, respectively[25,26]. Although both are macrolide antibiotics, azithromycin has a longer half-life due to its

	Time after drug infection (hour)					
Antibiotics	**2**	**4**	**6**	**9**	**24**	**logP**
Clarithromycin	5.4 ± 1.3	6.4 ± 0.5	12.5 ± 3.6	8.5 ± 2.4	16.2 ± 4.5	3.2
Azithromycin	2.8 ± 1.3	4.6 ± 1.4	9.4 ± 1.5	13.1 ± 3.0	44.7 ± 8.2	3.0
Rifampicin	15.8 ± 4.4	19.7 ± 5.7	47.0 ± 11.3	31.4 ± 4.4	NA	2.7
Ethambutol	1.6 ± 0.31	5.1 ± 3.8	13.3 ± 4.8	10.4 ± 3.6	NA	0.4
Clofazimine	11.5 ± 4.2	13.1 ± 0.8	25.2 ± 5.8	20.8 ± 5.0	33.0 ± 7.7	7.7
Amikacin	0.4 ± 0.05	0.4 ± 0.04	0.4 ± 0.03	0.5 ± 0.01	0.6 ± 0.1	-8.8

Table 4. Fat body/hemolymph concentration ratio. Data are shown as the mean ± standard deviation. *NA* not available, *logP* logarithm of *n*-octanol/water partition coefficient.

Antibiotics	Regimen	Cmax (μg/mL)	AUC (μg hour/mL)
Clarithromycin	Single dose	13.6 ± 3.0	477.5 ± 62.9
	After five days of rifampicin treatment	10.4 ± 1.3	452.4 ± 76.0
Azithromycin	Single dose	41.2 ± 9.1	410.6 ± 37.8
	After five days of rifampicin treatment	43.4 ± 5.4	371.2 ± 23.3
Rifampicin	Single dose	5.4 ± 2.4	18.1 ± 7.0
	After five days of clarithromycin treatment	4.3 ± 1.5	15.1 ± 5.3
	After five days of azithromycin treatment	4.2 ± 1.6	14.5 ± 5.1
Ethambutol	Single dose	19.7 ± 1.2	94.1 ± 9.4
	After five days of clarithromycin treatment	23.9 ± 2.4	100.5 ± 16.7
	After five days of azithromycin treatment	30.0 ± 5.9	122.6 ± 34.8
	After five days of rifampicin treatment	25.5 ± 3.6	94.0 ± 19.9
Clofazimine	Single dose	2.3 ± 0.19	79.6 ± 6.9
	After five days of clarithromycin treatment	2.0 ± 0.52	85.1 ± 28.5
	After five days of azithromycin treatment	1.4 ± 0.36	107.1 ± 35.2
	After five days of rifampicin treatment	1.4 ± 0.11	106.1 ± 15.5

Table 5. Antibiotics exposures with or without combination drugs. Data are shown as the mean ± standard deviation. *Cmax* maximum concentration, *AUC* area under the concentration–time curve.

superior tissue penetration[26]. In contrast, the half-life of clarithromycin in silkworms was approximately three times longer than that of azithromycin (Table 1). Additionally, while the half-life of clofazimine in humans has been reported to be 864 hours[27], its half-life in silkworms was only 30 h. Furthermore, amikacin hardly binds to proteins in human blood, and its half-life is reported to be as short as 3.4 hours[28]. However, in silkworm blood, more than 70% of amikacin binds to proteins (Table 3), and it was found that it is hardly eliminated from the silkworm's system. Contrary to the findings of the previous report[10], this study discovered that there are certain drugs for which predicting pharmacokinetics in humans based on their pharmacokinetic properties in silkworms is difficult. One new finding was that ethambutol, which is eliminated from the silkworm with a short half-life similar to humans[30]. This particular drug was not investigated by Hamamoto et al., who only focused on drugs metabolized by cytochrome P450 in silkworms[10]. It remains unclear whether drugs that are not metabolized and excreted in urine are eliminated from silkworms. Since this study did not delve into the specifics of the drug elimination pathway in silkworms, further investigation is needed to clarify the differences in pharmacokinetics between humans and silkworms. Nonetheless, the objective of the current study was to establish the dose and exposure relationship in silkworms, which has been achieved.

Although the 5th instar silkworm weight growth more than doubled within five days, pharmacokinetic parameters other than those for amikacin were comparable between 5th instar-day 1 and -day 5 silkworms (Table 1). In contrast, amikacin showed large variations in the parameter estimates for total clearance and area under the concentration–time curve in both single-dose and dose proportionality studies. This was mainly due to the lack of adequate data to estimate amikacin clearance. Pharmacokinetic parameter estimation performance depends on data adequacy for model building, and estimating the total clearance requires drug concentration data in the late elimination phase[15]. However, amikacin was barely eliminated from the silkworms; hence, its total clearance could not be accurately estimated.

For clofazimine, the maximum concentration remained almost constant, even though the dose increased, in contrast to the area under the concentration–time curve (Table 2). Clofazimine was in a saturated state during treatment because of its low solubility in water[16], which could explain these results. In fact, when clofazimine was dissolved in saline solution and administered to the silkworms, it remained in suspension.

Except for amikacin, all antibiotics were eliminated from the silkworms. Clarithromycin and azithromycin are mainly metabolized by cytochrome P450 enzymes in humans[17], and p-glycoprotein, a typical drug efflux transporter, has been suggested to be associated with clofazimine elimination in humans[16,18]. Although cytochrome P450 and some ATP-binding cassette transporters have been identified in silkworms[10,19], the metabolism and excretion of these drugs via transporters were not distinguished in this study. Moreover, because little is known about the elimination of hydrophilic compounds, such as ethambutol and amikacin, it is difficult to explain the large half-life difference measured between amikacin and ethambutol (Table 1) and to which protein amikacin was bound (Table 3). In humans, these hydrophilic drugs are mainly excreted in urine[20]; however, silkworms do not urinate until they become moths. Therefore, further investigation of the hydrophilic drug elimination routes used is required. Nonetheless, the purpose of the current study was not to clarify the elimination pathways of antibiotics, but rather the dose and exposure relationship in silkworms.

Rifampicin and clarithromycin, the well-known drug-metabolizing enzyme inducer and inhibitor in humans, respectively, showed no drug-drug interactions after five days of treatment in silkworms (Table 5). Effects of these interacting drugs have been reported to increase the intensity of the interaction in a time-dependent manner, taking approximately 40 days for rifampicin and 15 for clarithromycin[21,22]. However, the silkworm lifecycle may have been too short to observe these effects. In addition, because it is unknown whether these enzymes are induced in silkworms, further research on this aspect is required. Nevertheless, these results imply that pharmacokinetics do not change under anti-pulmonary MAC disease drug combination conditions in silkworms; hence, the relationship between antibiotic dosage and exposure can be simplified. This is advantageous for investigating the effects of antibiotics in combination with silkworm infection models.

This study revealed the pharmacokinetics of anti-pulmonary MAC disease drugs in silkworms, allowing the estimation of antibiotic exposure in silkworms from their dosages. These findings are necessary information for examining the relationship between antibiotic exposure and efficacy in a silkworm infection model. However, amikacin was not eliminated from silkworms; thus, it is considered unsuitable for evaluating antimicrobial effects in silkworm infection models. Furthermore, since this study was conducted in a state devoid of MAC infection, the impact of MAC infection on pharmacokinetics remains unclear. To clarify this point, future research is warranted using a silkworm infection model with MAC.

Methods

Animals and reagents

Silkworms, *Bombyx mori* (Hu•Yo × Tsukuba•Ne), were reared on eggs (Ehime Sanshu, Ehime, Japan) using artificial food (Silkmate 2S; Katakura Industries Co., Ltd. Tokyo, Japan) until growth to the 5th instar stage. Clarithromycin, rifampicin, amikacin, kanamycin, acetic acid, 1 mol/L ammonium acetate, LC/MS-grade acetonitrile, and dimethyl sulfoxide were purchased from FUJIFILM Wako Pure Chemical Co. (Tokyo, Japan). Azithromycin was purchased from Tocris Bioscience (Bristol, UK) and ethambutol from LKT Laboratories, Inc. (St. Paul, MN, USA). Clofazimine was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and phenacetine from Sigma-Aldrich (St. Louis, MO, USA). All other solvents and reagents used were of analytical grade.

Single-dose study

Fifty microliters of antibiotics dissolved in saline were injected separately into the 5th instar-day 1 and -day 5 silkworms using a 1-mL syringe attached to a 27-G needle, as described previously[10]. As 5th instar silkworm weight growth more than doubled, it was necessary to confirm whether the silkworm pharmacokinetic parameters

were uniform across the growth process. Doses per body weight of the antimicrobial agents were equivalent to those used in patients with pulmonary MAC disease[1]; 10 mg/kg clarithromycin and azithromycin, 20 mg/kg rifampicin, ethambutol, and amikacin, and 2 mg/kg clofazimine. Hemolymph was collected by cutting the first leg, as previously described[10]. The sample collection times were 1, 2, 4, 6, and 9 h after antibiotic injection for clarithromycin, azithromycin, clofazimine, and amikacin, and 2, 4, 9, 24, and 48 h after antibiotic injection for rifampicin and ethambutol. Except for ethambutol, hemolymph samples were collected consecutively from the same silkworm (N = 3 for each antibiotic). Ethambutol samples were collected from different silkworms during each sample collection period (N = 5 for each sampling point). As ethambutol had a small distribution volume, its pharmacokinetic parameters were affected by the amount of hemolymph shed. Clofazimine was injected as a saline suspension because of its low water solubility[21]. Hemolymph samples were stored at -80℃ until quantification.

Dose proportionality study

Two additional dose studies were conducted to confirm dose proportionality of the antibiotics. Each drug dose was summarized as follows: 5, 10, and 20 mg/kg clarithromycin, azithromycin, and amikacin; 10, 20, and 40 mg/kg rifampicin and ethambutol; and 2, 4, and 10 mg/kg clofazimine. Fifth instar-day 5 silkworms were used in this study. The sample collection time and hemolymph collection methods were the same as those used in the single-dose study.

Protein-binding rates of antibiotics in silkworm hemolymph

An ultrafiltration method was chosen to determine the hemolymph protein-binding rate of antibiotics using Vivacon 500 centrifugal units (10,000 molecular weight cutoff; Sartorius AG, Göttingen, Germany). Antibiotics were individually injected into 5th instar silkworms in three separate doses (0.1, 1, and 10 mg/kg; N = 3 each) and incubated at 25℃ for 2 h to reach a protein-binding equilibrium for the antibiotics. Then, 400 µL hemolymph containing each antibiotic was collected and placed in ultrafiltration tubes, followed by centrifugation at 15,000 × *g* for 15 min at 25℃. The total hemolymph (C1) and concentrate (C2) concentrations were measured, and the protein-binding rate calculated according to the following formula:

$$\text{Protein} - \text{binding rate} = 1 - \text{C2}/\text{C1}$$

The filter permeability of each antibiotic was examined in a preliminary study. The hemolymph was filtered using an ultrafiltration filter to obtain polymer-free hemolymph. Filter permeability was examined by dissolving the drug in polymer-free hemolymph and passing it through another ultrafiltration filter. Antibiotics, other than clofazimine, were not absorbed by the filters. In contrast, clofazimine was almost completely absorbed by the filter; thus, its protein-binding rate was not evaluated.

Fat body transferability

The acid-fast bacterium *M. abscessus*, has been reported to proliferate in the fat bodies of silkworms[22]. Therefore, we investigated the transferability of antibiotics from hemolymph to fat bodies. The antibiotics were injected into 5th instar silkworms at a dose of 10 mg/kg. Hemolymph was collected 2, 4, 6, 9, and 24 h after injection. Simultaneously, the silkworms were euthanized via immersion in 70% ethanol for 3 min, and fat bodies thereafter collected (N = 3 for each sampling point). The collected fat bodies were homogenized and stored at -80℃ until quantification. Transfer rate to the fat bodies was calculated by dividing the concentration in the fat bodies with that in the hemolymph.

Drug-drug interactions

As anti-pulmonary MAC disease drugs include rifampicin, a well-known drug-metabolizing enzyme inducer[23], and clarithromycin, an inhibitor[24], their drug-drug interactions have been tested in silkworms. Interacting drug candidates, such as rifampicin, azithromycin, and clarithromycin, were administered at 10 mg/kg on consecutive days from days 1–5 of silkworm growth. Then, anti-pulmonary MAC disease drugs were injected into 5th instar-day 5 silkworms at the same dose as that used in the single-dose study. Drug-drug interactions were examined by comparing exposure between the single and concomitant use treatments. As clarithromycin and azithromycin are not typically used concomitantly to treat this disease, the interaction between them was not investigated. Five silkworms were treated with each antimicrobial combination.

Quantification of drug concentrations and pharmacokinetic analysis

The concentrations of clarithromycin, azithromycin, ethambutol, rifampicin, and clofazimine were measured using a validated high-performance liquid chromatography-mass spectrometry method with minor modifications[21]. Briefly, samples were protein-precipitated using a fivefold volume of acetonitrile containing 5 µg/mL phenacetine as an internal standard and centrifuged at 15,000 × *g* for 10 min at 4℃. The supernatant (2 µL) was injected into a Shimadzu mass spectrometry system (LCMS2020) coupled to a Shimadzu high-performance liquid chromatography system (Shimadzu, Kyoto, Japan). Separation was performed in a Shim-pack Scepter C18-120 (2.1 mm i.d. × 100 mm) with a 5-µm particle size (Shimadzu) maintained at 40℃ and eluted with a mobile phase flow rate of 0.4 mL/min. The mobile phase consisted of 10 mM ammonium acetate buffer (pH 5.3) and acetonitrile under gradient conditions. The lower limit of quantification was 0.01 µg/mL for clarithromycin, 0.02 µg/mL for azithromycin, ethambutol, and clofazimine, and 0.05 µg/mL for rifampicin.

The amikacin concentration was also measured using a validated high-performance liquid chromatography-mass spectrometry method with minor modifications[25]. Briefly, a twofold volume of 10% (w/v) trichloroacetic

acid containing 10 μg/mL kanamycin as an internal standard was added to the sample. Thereafter, samples were cooled at -20℃ for 10 min, vortexed again, and centrifuged at 15,000 × *g* for 10 min at 4℃ to precipitate proteins. The supernatant was mixed with an equal volume of 1 mol/L ammonium formate and 2 μL injected into the Shimadzu mass spectrometry system (LCMS2020) coupled to a Shimadzu high-performance liquid chromatography system. Separation was performed in a Shim-pack GIST-HP 3-μm Amide Metal free column (2.1 mm i.d. × 50 mm; Shimadzu Co.) maintained at 40℃ and eluted with a mobile phase flow rate of 0.3 mL/min. The mobile phase comprised a 0.1% (v/v) formic acid-1.0% (v/v) ammonium formate solution and acetonitrile under gradient conditions. The lower limit of quantification was 5 μg/mL.

The recovery was greater than 93%, and the within- and between-day coefficients of variation within 15%. No matrix effects or carryover were observed.

Data analysis

Non-compartment analysis was used to calculate the pharmacokinetic parameters, such as maximum concentration, area under the concentration–time curve, total clearance, and volume of distribution at steady-state using Phoenix WinNonlin version 8.3 (Pharsight Co., Mountain View, California, USA). Antibiotic concentration–time curves were drawn using R software (version 4.1.1; https://www.R-project.org).

Data availability

The datasets generated in this study are available from the corresponding author upon reasonable request.

Received: 20 October 2023; Accepted: 22 July 2024
Published online: 23 July 2024

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Acknowledgements

This study was supported in part by a Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan (no. N202704), the Joint Research Program in Meiji Pharmaceutical University (JRP22MPU), and the Research Institute of Tuberculosis to F.W. This study was supported in part by grants from the Japan Agency for Medical Research and Development/Japan International Cooperation Agency (AMED) to Y.H. (JP20fk0108064, JP20fk0108075, JP21fk0108093, JP21fk0108129, JP22jm0510004, JP22wm0225004, JP22wm0325003, JP22fk0108553, JP 22fk0108558, JP23fk0108608, JP23fk0108673, JP23gm1610003, JP23gm1610007, JP23wm0125007, JP23wm0225022, and JP23wm0325054,) and Y.M. (JP23wm0325054 and JP22fk0108553h0001); from the Japan Society for the Promotion of Science (JSPS) for International Collaborative Research to Y.H (JP63KK0138-A), for Scientific Research (C) to Y.H. (JP23K07665 and JP23K07958) and Y.M. (JP20K07022); and for Encouragement of Scientists to F.W. (JP24H02649).

Author contributions

Study conception: F.W. and Y.M. Data collection and analysis: F.W. Manuscript drafting: F.W., Y.M., and K.H. Study supervision: Y.M., Y.H., and S.M. Critical revision: Y.M., T.S., Y.M., Y.H., S.M., and K.H. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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