Brief Report

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Anti-*Mycobacterium avium* complex activities of streptcytosine analogs from a marine actinomycete as nucleoside antibiotics

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SUMMARY: To examine the potential of nucleoside antibiotics as therapeutic agents against *Mycobacterium avium* complex (MAC), the *in vitro* and *in vivo* anti-MAC activities of streptcytosine A (**1**), plicacetin (**2**), and bamicetin (**3**) derived from a marine actinomycete were evaluated. Compounds **1**–**3** exhibited antimicrobial activities against *M. avium* and *M. intracellulare*, with minimum inhibitory concentration values of 4.0 and 16 μg/mL, respectively, as assessed by the microdilution method. In silkworm infection models of *M. avium* and *M. intracellulare*, these compounds also exhibited therapeutic efficacies at lower doses than clarithromycin, with 50% effective doses of between 7.6 and 28 μg/larva·g, and no toxicity was observed. A pharmacokinetic analysis further revealed elimination half-lives of 3.0, 2.3, and 5.1 hours, respectively, in the silkworm hemolymph. These results suggest the potential of **1**–**3** as lead candidates for the development of potent anti-MAC drugs.

Keywords: Streptcytosine, plicacetin, bamicetin, silkworm infection model, *Mycobacterium avium* complex (MAC), natural product

1. Introduction

Non-tuberculous mycobacterial (NTM) disease is an infectious disease that primarily affects the lungs, and its incidence has been increasing globally in recent years (*1,2*). Unlike tuberculosis, NTM disease is mainly caused by non-tuberculous mycobacteria found in environmental sources. Among these infections, *Mycobacterium avium* complex (MAC) infection, primarily caused by *M. avium* and *M. intracellulare*, accounts for more than 80% of NTM disease cases in Japan (*2*). The standard treatment for MAC infection consists of a multidrug regimen comprising macrolides, such as clarithromycin and azithromycin, in combination with rifampicin and ethambutol. However, despite prolonged administration exceeding 6 months, the attenuation of symptoms is often inadequate due to the emergence of drug-resistant strains, rendering the disease refractory. In such cases, injectable aminoglycosides or amikacin liposome inhalation suspensions are recommended as adjunctive therapy. Nevertheless, an optimal treatment regimen for MAC infection has yet to be established. Therefore, the development of novel anti-MAC drugs remains an urgent medical priority.

In the search for novel drug candidates, a key requirement is the presence of a unique structure that has not been previously identified. Nucleoside compounds, such as mavintramycin and amicetin, were recently reported to exhibit anti-MAC activity (*3*), underscoring the potential of nucleoside antibiotics as promising anti-MAC agents. Therefore, we focused on nucleoside antibiotics, specifically streptcytosine analogs, which were previously identified as antimicrobial agents against *M. smegmatis* during our screening of anti-tuberculosis compounds derived from marine invertebrates and microorganisms (*4*). In the present study, we examined the anti-MAC activities of streptcytosine analogs isolated from the marine actinomycete *Streptomyces* sp. TPU1236A using *in vitro* and *in vivo* assays, namely, the liquid microdilution method and silkworm infection model, respectively. In addition, their elimination half-lives ($t_{1/2}$) in the silkworm hemolymph were assessed by high-performance liquid chromatography (HPLC).

2. Materials and Methods

2.1. Materials

Streptcytosine A (**1**), plicacetin (**2**), and bamicetin (**3**) were purified from the culture broth of marine-derived *Streptomyces* sp. TPU1236A (*4*). Rifampicin was obtained from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan), and clarithromycin was purchased from Tokyo Chemical Industries (Tokyo, Japan).

2.2. Microorganisms

M. avium JCM 15430 and *M. intracellulare* JCM 6384 were obtained from the Japan Collection of Microorganisms, RIKEN BRC, which is part of the National BioResource Project of MEXT, Japan.

2.3. Measurement of minimum inhibitory concentration (MIC) values using the liquid microdilution method

The MIC values of **1**–**3** against *M. avium* and *M. intracellulare* were evaluated using the liquid microdilution method according to a previously established protocol (*5,6*). *M. avium* and *M. intracellulare* were cultured at 37°C for seven days in Middlebrook 7H9 broth (1.04% Middlebrook 7H9 broth, 0.05% Tween 80, 0.5% bovine serum albumin, 0.2% glucose, and 0.085% NaCl) until reaching approximately 1.0×10^9 colony-forming units (CFU)/mL. Bacterial cultures were then diluted 500-fold with the same fresh broth. A 95-µL aliquot of the diluted suspension was dispensed into each well of a 96-well microplate with or without test samples (5 µL in methanol). The microplate was incubated at 37°C for seven days. Turbidity was assessed by measuring absorbance at 550 nm using a spectrophotometer. MIC was defined as the lowest concentration of the test compound that inhibited bacterial growth by 90% of the control (without the compound).

2.4. Evaluation of the 50% effective dose (ED_{50}) in the silkworm infection model

The silkworm infection model was conducted following a previously established protocol (*5,7–14*). Fertilized silkworm eggs of *Bombyx mori* (Hu·Yo × Tukuba·Ne) were obtained from Ehime Sansyu (Ehime, Japan) and reared on an artificial diet (Silk Mate 2M; Nihon Nosan Kogyo, Kanagawa, Japan) in an incubator at 27°C until the fourth-instar larval molting stage. On the first day of the fifth-instar larval stage following molting, silkworms were fed the artificial diet until their body weight reached approximately 2 g. On the next day, a suspension of *M. avium* or *M. intracellulare* (2.5×10^7 CFU/larva·g in 50 µL Middlebrook 7H9 broth) was injected into the hemolymph of silkworm larvae (2.0 g, $n = 5$) using a disposable 1-mL syringe (NIPRO, Osaka, Japan) equipped with a 27-G needle (TERUMO, Tokyo, Japan). Within 30 minutes of infection, test samples (50 µL in saline or 10% DMSO) were administered *via* injection. After the injection, silkworms were maintained at 37ºC without feeding, and their survival rate was monitored for 96 hours post-injection. ED_{50} values were defined as the dose required to achieve a 50% survival rate, normalized per gram of silkworm body weight.

2.5. Evaluation of drug metabolism in silkworm larvae

Drug metabolism in silkworm larvae was assessed according to a previously established protocol (*15*,*16*). Hemolymph samples were collected at the time points specified in the figure legends after injecting **1**–**3** (50 µL of 1 mg/mL) directly into the hemolymph. The collected hemolymph (50–100 µL) was mixed with an equal volume of acetonitrile, followed by centrifugation at 10,000 rpm at 4°C for 5 min. The resulting supernatant was analyzed by HPLC under the following conditions: column, SUPELCO Express C18 (2.1 mm × 50 mm, Sigma-Aldrich Chemical Company, St. Louis, MO, USA); mobile phase, acetonitrile with a 0.1% formic acid gradient (5–95% over 10 min); flow rate, 0.4 mL/min; column temperature, 50ºC; injection volume, 10 µL; detection, UV at 320 nm.

3. Results and Discussion

The *in vitro* activities of **1**–**3** against *M. avium* and *M. intracellulare* were evaluated using the liquid microdilution method, and their MIC values are summarized in Table 1. Compounds **1**–**3** exhibited antimicrobial activities against *M. avium*, each with an MIC value of 4.0 µg/mL. They also showed antimicrobial activities against *M. intracellulare* with consistent MIC values of 16 µg/mL. In our previous study, **1**–**3** were isolated as antimicrobial compounds that were active against *M. smegmatis* and identified as nucleoside antibiotics containing cytosine, amosamine, amicetose, and *p*-aminobenzoic acid (PABA) (Figure 1) (*4*). The findings obtained demonstrated that streptcytosines B–E, which lack amosamine and PABA, did not exhibit anti-*M. smegmatis* activity, suggesting that the presence of amosamine and PABA, whether individually or together, is essential for this activity.

Hosoda *et al*. (*3*) reported that the structurally related compounds, mavintramycin and amicetin, exhibited

Table 1. MIC and ED_{50} values of 1-3 against *M. avium* and *M. intracellulare*

	M. avium		*M. intracellulare*	
	MIC (µg/mL)	ED_{50} (µg/larva·g)	MIC (µg/mL)	ED_{50} (µg/larva·g)
Streptcytosine A (**1**)	4.0	9.1	16	28
Plicacetin (**2**)	4.0	15	16	26
Bamicetin (**3**)	4.0	7.6	16	12
Clarithromycin	0.098	23	0.012	42

anti-MAC activities. Since mavintramycin lacks PABA, this finding indicates that the amosamine moiety is critical for anti-NTM activity. In the present study, **1**–**3**, which contain cytosine, amosamine, and amicetose, similar to amicetin and mavintramycin, exhibited anti-MAC activities. Mavintramycin A has been shown to inhibit *M. avium* by binding to 23S ribosomal RNA and interfering with protein synthesis (*3*). Furthermore, a crystallographic analysis revealed that amicetin bound to the 70S ribosomal subunit of *Thermus thermophilus*, occupying the P-site in the peptidyl transferase center (*17*), which differs from the binding sites of clinically used antibiotics, such as clarithromycin and amikacin. Therefore, **1**–**3** may interact with similar ribosomal sites in *M. avium* and *M. intracellulare*, potentially conferring efficacy against drug-resistant clinical strains.

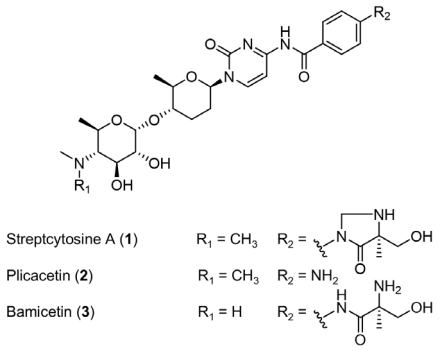

Figure 1. Structures of 1-3.

The therapeutic efficacies of **1**–**3** were further evaluated using silkworm infection models of *M. avium* and *M. intracellulare* (n = 5). These models closely mimic *in vivo* conditions and are useful for examining the therapeutic efficacies and pharmacokinetics of antimicrobial agents, similar to murine models, while requiring minimal sample quantities and enabling rapid evaluations (*15,18*). As shown in Figure 2 and Table 1, the administration of **1**–**3** to silkworms infected with *M. avium* resulted in dose-dependent therapeutic

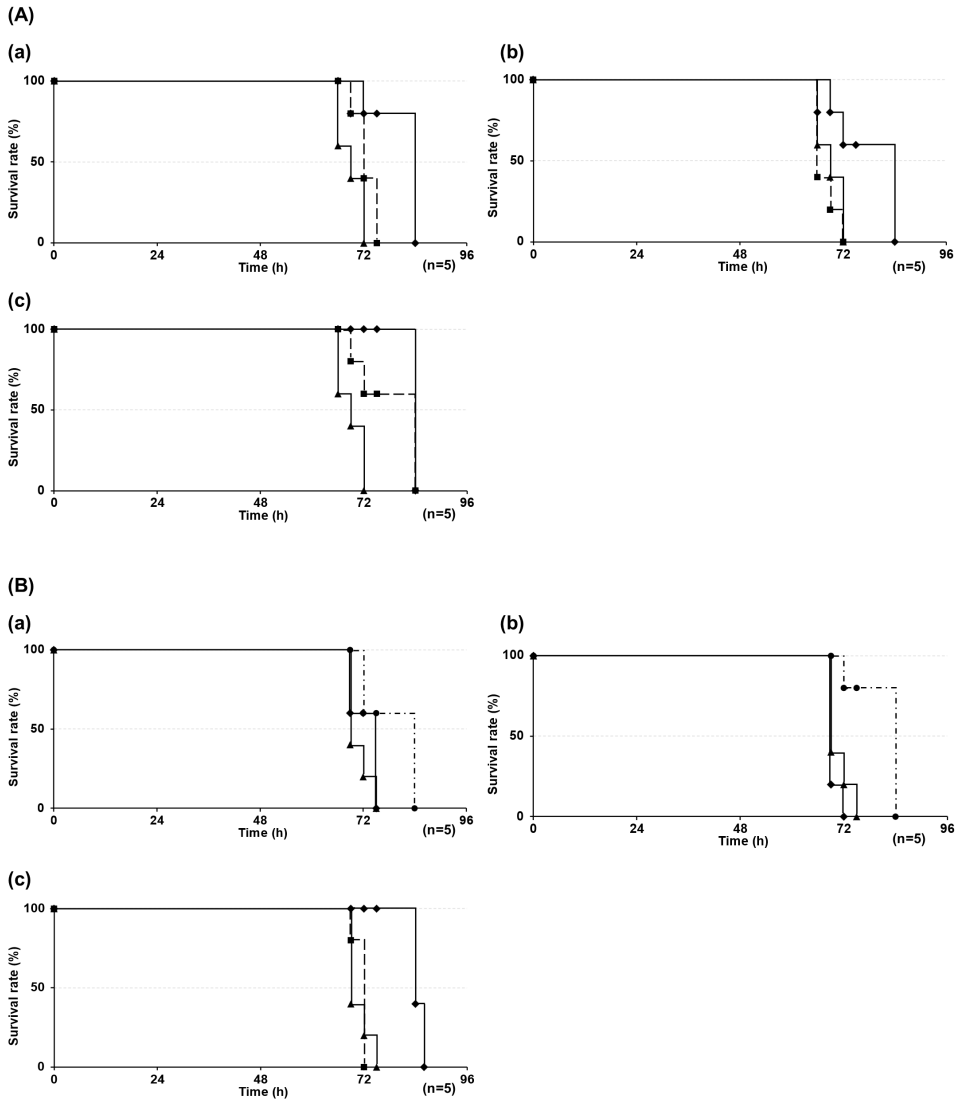

Figure 2. Therapeutic effects of 1–3 in silkworm infection models of (A) *M. avium* and (B) *M. intracellulare*. (a) Streptcytosine A (**1**), (b) plicacetin (**2**), and (c) bamicetin (**3**). ●: 32, ♦: 16, ■: 8.0, ▲: 0 μg/larva·g. Experiments were performed twice, and reproducible data were obtained.

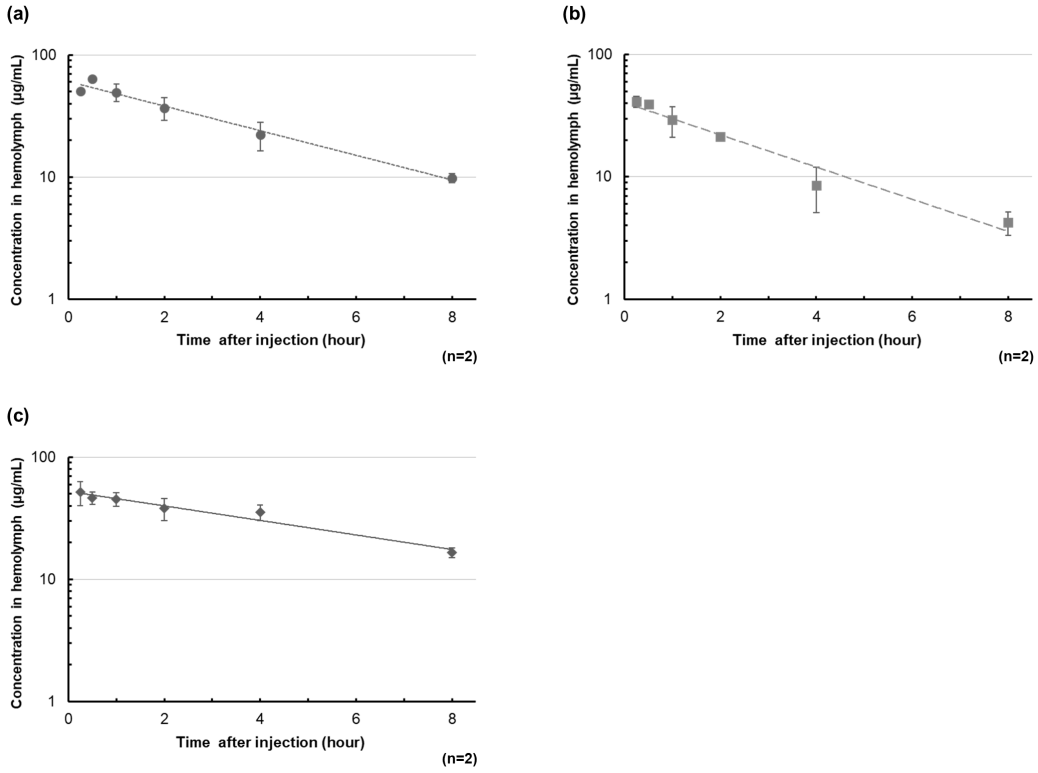

Figure 3. Time-dependent concentration profiles of 1–3 in the silkworm hemolymph. ●: Streptcytosine A (**1**) ($t_{1/2}$ = 3.0 hours) (a); ■: plicacetin (**2**) ($t_{1/2}$ = 2.3 hours) (b); ♦: bamicetin (**3**) ($t_{1/2}$ = 5.1 hours) (c). The silkworm hemolymph (n = 2) was collected 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 hours after the injection of **1**–**3**. Experiments were performed twice, yielding consistent results.

effects, with ED_{50} values of 9.1, 15, and 7.6 μg/larva·g, respectively. Similarly, in the *M. intracellulare*-infected silkworm model, the corresponding ED_{50} values were 28, 26, and 12 μg/larva·g, respectively. Moreover, none of the compounds exhibited toxicity towards silkworms within a 72-hour observation period (data not shown). These results indicate that **1**–**3** exhibited potent *in vivo* anti-MAC activities, with ED_{50} values that correlated with their MIC values. Since mavintramycin A was previously shown to exhibit therapeutic efficacy in a murine *M. avium* infection model (*3*), **1**–**3** may also be effective in mammalian systems. Moreover, the ED_{50} values of clarithromycin in the silkworm models of *M. avium* and *M. intracellulare* were 23 and 42 μg/larva·g, respectively, which were markedly higher than those of **1**–**3**, suggesting their strong potential as anti-MAC agents.

A pharmacokinetic analysis was conducted by collecting the silkworm hemolymph at various time points after the administration of **1**–**3** and subjecting it to a HPLC analysis (*15*). The $t_{1/2}$ of **1**–**3** were 3.0, 2.3, and 5.1 hours, respectively (Figure 3). These moderate $t_{1/2}$ are consistent with their therapeutic efficacies observed *in vivo*, suggesting that **1**–**3** maintain pharmacologically active concentrations in the hemolymph during the dosing interval. Moreover, these values were within the typical range reported for clinically used antimicrobial agents in the silkworm model (*19*). This pharmacokinetic profile supports their potential as anti-MAC agents. To the best of our knowledge, this is the first study to investigate the pharmacokinetics of these related compounds, and the results obtained provide valuable insights for future *in vivo* studies.

In conclusion, the present study demonstrated that nucleoside antibiotics, specifically streptcytosine analogs derived from a marine actinomycete, exhibited significant anti-MAC activities both *in vitro* and *in vivo*. Future research needs to focus on optimizing these compounds through *in vivo* structure-activity relationship studies, particularly by investigating structurally related compounds, such as amicetin and mavintramycins. In addition, their potential for synergistic effects in combination with existing clinical drugs merits further research to accelerate the development of novel anti-MAC therapeutics.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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