Original Article

Evaluation of *in vitro* inhibitory effects of prumycin on the growth of *Babesia* and *Theileria* parasites

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Abstract:

Prumycin is a carbohydrate antibiotic. It was isolated from a *Streptomyces* sp. at Kagawa Prefecture, Japan. It has antifungal, antitumor, and antimalarial activities. The inhibitory properties of prumycin were evaluated *in vitro* cultures of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*; furthermore, the *in vitro* drug combination with clofazimine was assessed for *Babesia bovis* and *Babesia caballi.* The IC_{50} values of prumycin were 22.3, 0.96, 1.89, and 21.17 µM for *B. bovis, B. bigemina, B. caballi*, and *T. equi*. The combination of prumycin and clofazimine had a synergetic action on *Babesia* parasites that improved the potency and decrease the possible toxic side effect in *B. caballi* and *B. bovis* cultures. Therefore, prumycin might be of value in blended therapy of babesiosis and theileriosis and further studies are required to evaluate its *in vivo* effects.

Keywords: Prumycin; *Babesia*; *Theileria equi*; *In vitro*; Clofazimine; Combination

INTRODUCTION

Babesia, tick-borne parasite, infects erythrocytes in animals and humans worldwide. The clinical symptoms include malaise, fever, hemolytic anemia, hemoglobinuria, icterus, and edema. *Babesia bovis* infected cattle will die from obstruction of brain blood vessels **(Mamoun and Allred 2018).**

Babesia microti and *B. diverigens* are zoonotic for human in the united states of America and in Europe, respectively. A number of diseases can occur in patients from severe disease to asymptomatic infection **(Vannier et al., 2008)**.

Babesia infections worldwide have caused severe profitable losings in livestock production **(Kuttler, 1988; Mamoun and**

Allred 2018). *Theileria equi* causes piroplasmosis in equines and affects its world trade. Babesicidal drugs were ineffective due to the development of resistance or toxicity **(Upcroft, 1994; Vial and Gorenflot, 2006).** Therefore, the

 development of new drugs that have reduced poisonousness to the hosts is desired.

Prumycin (4-N-[D-alanyl]-2,4-diamino-2,4-dideoxy-L-arabinose), a carbohydrate antibiotic **(Omura et al., 1974; Ōmura et al., 1972)**, was isolated from a *Streptomyces* sp. at Kagawa Prefecture, Japan **(Hata et al., 1971; Ōmura et al., 1973)**. Prumycin inhibits protein synthesis in fungi **(Schwartz et al., 1974)** and DNA and protein synthesis in Hela S3 cells **(Okubo et al., 1980b)**. It is also known to inhibit the cytosolic alanyl aminopeptidase (AAP-S) from human liver cytosol and aminopeptidase N from human seminal plasma **(Yamamoto et al., 2000)**. A high throughput virtual screening showed that prumycin inhibit *Brucella melitensis* methionyl-tRNA-synthetase **(Kumari et al., 2017)**

Prumycin has antifungal **(Hata et al., 1971; Ōmura et al., 1973; Tanaka et al., 2017)**, antitumor **(Okubo et al., 1980a; Okubo et al., 1979; Okubo et al., 1980b, c)**, and antimalarial **(Otoguro et al., 2004)** activities. *Babesia* and *Plasmodium falciparum* have similarities as intraerythrocytic apicomplexan parasites. The purpose of the research was thus to evaluate prumycin *in vitro* inhibitory effects on *Babesia* and *T. equi* parasites.

MATERIALS AND METHODS

1. Chemical reagents

Prumycin and clofazimine were purchased from Sigma-Aldrich (Tokyo, Japan). The different materials were bought (Wako Pure Chemicals, Osaka, Japan). Stock solutions of 100 mM (Clafozimine) and 20 mM in DMSO were prepared and stored at−30°C until use. Diminazene aceturate (GANASEG) was purchased from (Ciba-Geigy Japan Lit., Tokyo, Japan) and used as a comparator drug. A working stock solution of 10 mM melted in purified water was prepared and stored at −30◦C until required for use. The solvents were melted and applied to cultures at concentrations near the highest drug levels in the treated cultures as negative controls.

2. *In vitro* cultivation of *Babesia* and *Theileria* parasites

Prumycin was evaluated for its chemotherapeutic effect against *B. bovis* (Texas strain) **(Hines et al., 1992)**, *B. bigemina* (Argentina strain) **(Jorgensen et al., 1992)**, and *B. caballi* **(Avarzed et al., 1997)** and *T. equi* **(Aboulaila et al., 2010a)** USDA strains. Bovine and equine red blood cell parasites were cultivated by using an incessant method of stationary phase microaerophilic cultivation **(Aboulaila et al., 2010a; Igarashi et al., 1998)**. The culture medium, M199, applicable to *B. bovis*, *B. bigemina*, and *T. equi* (acquired from Sigma-Aldrich, Tokyo, Japan), was enhanced with 40% either bovine or equine sera and 60 IU/ml of penicillin G, 0.15 µg/ml of amphotericin B, and 60 µg/ml of streptomycin (Sigma-Aldrich) and added to culture the parasites **(AbouLaila et al., 2014)**. The RPMI1640 medium (Sigma-Aldrich, Tokyo, Japan), supplemented with 40% equine serum, was used for *B. caballi* culture.

3. *In vitro* growth inhibition assay

The *in vitro growth* inhibitory test was embraced from previous studies **(Aboulaila et al., 2012; Igarashi et al., 1998)**. For all the parasites, the drug assessment parasite cultures have been modified to 1% from

cultures of 5% parasitemia using fresh RBCs. The growth-inhibitory test was completed in 96-well plates. Twenty microliters of the parasite bovine red blood cell mixture were apportioned per well together with 200 µl of

the culture medium having the demonstrated medication concentration based on a preliminary study. Prumycin concentrations of 5, 10, 25, 50, 100 µM for B. bovis, 0.1, 0.5, 1, 2, and 5 µM for *B. bigemina,* 0.5, 1, 2, 5 µM for *B. caballi,* and 5, 10, 25, 50, 100, and 200 µM for *T. equi* were tested. For the control, similar cultures without the drug and others having just the solvents at the most elevated concentration utilized were readied. For each parasite species, the trials were implemented three times per drug concentration, and for three different trials. Diminazene aceturate was compared with prumycin at 1, 5, 10, 50, 100, 1000, 2000 nM **(Matsuu et al., 2008)**, respectively. Cultures have been incubated at 37 °C, 5% CO_2, 5% O_2, and 90% N_2 atmosphere. For four days, 200 µl of the new medium inclosing the same drug concentration was substituted daily with the culture medium. In Giemsa-stained thin erythrocyte smear, the parasitemia was tracked using approximately 1000 erythrocytes. Changes were matched to the control with light microscopy for the morphology of the handled Babesia parasites. On the 3rd day, interpolation using a curve-fitting technique was used to calculate the value of 50 percent inhibitory concentration (IC_{50}) **(Aboulaila et al., 2010a)**.

4. Viability test

After four days of the treatment, 14 µL of parasite-free bovine RBCs was supplementary to 6 µL of the formerly drug-cured cultures in 200 µl of a new

growth medium without the medication. The new medium was substituted regularly for the next 10 days and the recrudescence of parasite was assessed after the medication was withdrawn **(Aboulaila et al., 2010a)**.

5. Drug combination test

Combination therapies of prumycin and clofazimine were tested in the *in vitro* cultures of *B. bovis* and *B. caballi.* Clofazimine was used at concentrations of 1, 2, 5, 10, and 25 µM **(Tuvshintulga et al., 2016)** to determine suitable concentrations for combination. Clofazimine and prumycin combinations (CF1P1, CF2P1, CF3P1, CF4P1, CF1 P2, CF2 P2, CF3 P2, and CF4 P2) for *B. caballi* and (CF1 P4, CF2 P4, CF3 P4, and CF4 P4) for *B. bovis* were prepared as previously described **(Aboulaila et al., 2010a)** with some modifications and based on *in vitro* inhibition assay of prumycin and clofazimine.

In combination, the dosage of each drug used was not harmful to the parasites. The simultaneous application of concentrations of clofazimine / prumycin to cultures was: for *B. bovis* (1/4, 2/4, 3/4, and 4.3/4 µM) and *B. caballi* (1/0.9, 2/0.9, 3/0.9, 4.3/0.9, 1/1.8, 2/1.8, 3/1.8, 4.3/1.8 µM), respectively. The effect was evaluated as previously recorded **(Salama et al., 201)**.

6. Effect of prumycin on host erythrocytes

The toxicity of prumycin to host erythrocytes was evaluated as previously described **(Aboulaila et al., 2010c)**. Bovine and equine RBCs were incubated in the existence of 100 µM prumycin (the highest concentration used) for 3 hours at 37 ° C; at that point erythrocytes were washed 3 times with medication free-media and utilized for the

development of *Babesia* parasites for 72 hours. The control untreated cells dealt with as the pre-treated cells. The form of parasite development in pre-treated erythrocytes was watched and contrasted with control untreated cells utilizing a light microscope.

7. Statistical analysis

The differences in the percentage of parasitemia for the *in vitro* cultivations were analyzed with JMP statistical software (SAS Institute Inc., USA) using the student's *t*-test. Statistically significant was a *P* value of < 0.05 for *in vitro* studies.

RESULTS

In vitro growth inhibition assay

Prumycin significantly (*P* < 0.05) inhibited the growth of the parasites at 50 µM *B. bovis* (Fig. 1A), 0.5 µM *B. bigemina* (Fig.1B), 1 µM *B. caballi* (Fig. 1C), and 10 µM *T. equi* (Fig. 1D). Prumycin suppressed the growth of *B. caballi* and *B. bigemina, B. bovis*, and *T. equi* in the presence of 5 µM, 50 µM, and 100 µM, respectively. We observed a significant *in vitro* development restraint of all *Babesia* species at five nM diminazene aceturate except day 1 was only significant at 100 nM. A concentration of 2000 nM resulted in complete suppression of treated parasites except for *B. caballi* that need a lower dilution of 50 nM to suppress the development (data not shown).

Complete parasites clearance was seen at 5 µM on the third (*B. caballi*), 50 µM on the fourth (*B. bovis*), 5 µM on the fourth (*B. bigemina*), and 200 µM on the fourth (*B. equi*) days of prumycin

treatment. After treatment, parasites cultured in drug-free medium for 10 days exhibited no regrowth of the parasites at 5 µM (*B. caballi*), 10 µM (*B. equi*), 2 µM (*B. bigemina*), and 50 µM (*B. bovis*) (Fig. 1). Lower drug concentrations resulted in the re-growth. There was no renewal for parasites treated with diminazene aceturate concentrations of 500 nM (*B. bovis*, *B. bigemina*, and *T. equi*) and 25 nM (*B. caballi*) (data not are shown). Prumycin and diminazene IC_{50} values for different *Babesia* species were presented (Table1). The addition of the solvents, DMSO and DDW, to the culture had no impact on the growth. Prumycin treatment resulted in degenerated parasites in the cultures of *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* as compared with non-treated parasites from DMSO negative control (not shown).

Drug combination test

Combination therapies of prumycin/ clofazimine for *B. caballi* resulted in significant inhibition at all the used combinations with inhibition of 87.5, 71.9, 80.5, 85.4, 86.5, 87, 91.7, and 93.3% for CF1P1, CF2P1, CF3P1, CF4P1, CF1 P2, CF2 P2, CF3 P2, and CF4 P2, respectively (Fig.2 A). The combinations of clofazimine/prumycin resulted in significant inhibition of *B. bovis* of 60.13, 58.5, 58, and 58% for CF1 P4, CF2 P4, CF3 P4, and CF4 P4, respectively (Fig.2 B).

Effect of prumycin on host erythrocytes

Prumycin was non-toxic to either bovine or equine erythrocytes at the highest concentration (100 µM) as similar parasitemias levels were recorded for pretreated and untreated erythrocytes (data not shown).

DISCUSSION

In the present study, prumycin inhibited the *in vitro* growth of *B. bovis*, *B. bigemina*, *B.*

caballi, and *T. equi*. DMSO did not affect the growth of the parasites; therefore, the growth inhibition observed in this study was due to the effects of prumycin. *B. caballi* and *B. bigemina* were more sensitive to prumycin than *B. bovis* and *T. equi.*

The IC_{50} values of prumycin for *Babesia* parasites were higher than the IC_{50} values of diminazene aceturate and clofazimine reported in this study. The IC_{50} values of prumycin for *Babesia* parasites were lower than those for *P. falciparum* **(Otoguro et al., 2004)**. The IC_{50} values of prumycin for *Babesia* and *Theileria* parasites were lower than atranorin **(Beshbishy et al., 2020)**, N-acetyl-L-cysteine **(Rizk et al., 2017)**, and fusidic acid **(Salama et al., 2013)**. The IC_{50} values of prumycin were in a similar range with enoxacin **(Omar et al., 2016)**, miltefosine **(AbouLaila et al., 2014)**, clotrimazole **(Bork et al., 2003c)**, tetracyclines **(Matsuu et al., 2008; Nott et al., 1990)**, purvalanol A **(Nakamura et al., 2007)**, and nerolidol **(AbouLaila et al., 2010b)**.

The IC_{50} values of prumycin were higher than the IC_{50} values of other tested antibabesial drugs such as quercetin **(AbouLaila et al. 2019c)**, apigenin and gallic acid **(AbouLaila, 2018)**, luteolin **(AbouLaila et al., 2019a)**, myrrh oil **(AbouLaila et al., 2020)**, and enrofloxacin **(AbouLaila et al., 2019b).**

The IC_{50} values of prumycin were higher than the IC_{50} values of other babesicidal drugs such as epoxomicin **(Aboulaila et al., 2010a)**, atovaquone (**Matsuu et al., 2008; Pudney and Gray, 1997)**, quinuronium sulfate **(Brockelman and Tan-ariya, 1991)**, imidocarb dipropionate **(Brasseur et al., 1998; Rodriguez and Trees, 1996)**, and clindamycin phosphate **(Brasseur et al., 1998)**.

The pretreatment of erythrocytes with the used concentrations of prumycin showed that it is non-toxic to the bovine and equine erythrocytes. Furthermore, the IC_{50} value of prumycin was 50 µM for aminopeptidase N from human seminal plasma **(Yamamoto et al., 2000)**. Moreover, neither change in the HeLa cell viability was observed after incubation with prumycin at 1840 µM (1, 000 µg /ml) nor suppression in mouse spleen cells at 115 µM (62.5 µg /ml). On the other hand, the IC_{50} values of prumycin were 6.61 µM (3.6 µg/ml) for the MRC-5 mammalian cells **(Otoguro et al., 2004)** and concentrations of > 9.2 µM (> 5 µg/ml) inhibited the growth of HeLa S-3 cells **(Okubo et al., 1980c)**. Therefore, prumycin could not be used alone for the treatment of babesiosis and combination with another drug might decrease its toxic side effect.

Combined drug treatment used to improve the effectiveness or reduce the toxicity of the drug. The combination of clofazimine and prumycin had an enhancing action on *Babesia* parasites that improved the potency and decrease the toxic dose in *B. caballi* and *B. bovis* cultures. Therefore, prumycin might be used in combination therapy.

The prumycin inhibits protein synthesis in fungi **(Schwartz et al., 1974)** and protein synthesis in Hela S3 cells **(Okubo et al., 1980b)**. furthermore, a high throughput virtual screening showed that prumycin inhibits *Brucella melitensis* methionyl-tRNA-synthetase **(Kumari et al., 2017)**. Methionyl-tRNA-synthetase is a main protein synthesis enzyme that combines codons with their respective amino acids considered as a central factor in the initiation of protein translation. **(Lee et al., 2004; Park et al., 2005)**. Interestingly, the methionyl-tRNA-synthetase genes are found in the gene bank

Table (1): IC_{50} values of prumycin and diminazene for *B. bovis*, *B. bigemina* , *B. caballi*, and *T. equi*

	IC_{50} (µM)[*]	
Organism	Prumycin	Diminazene
B. bovis	22.3 ± 1.1	0.34 ± 0.02
B. bigemina	0.96 ± 0. 3	0.17 ± 0.007
B. caballi	1.89 ± 0.1	0.009 ± 0.001
T. equi	21 ± 0.9	0.63 ± 0.03
P. falciparum[1]	16.5 ± 0.4[1]	ND

[*] IC_{50} values expressed as prumycin concentrations are in the micromolar of the growth medium and were determined using a curve- fitting technique from three separate experiments.
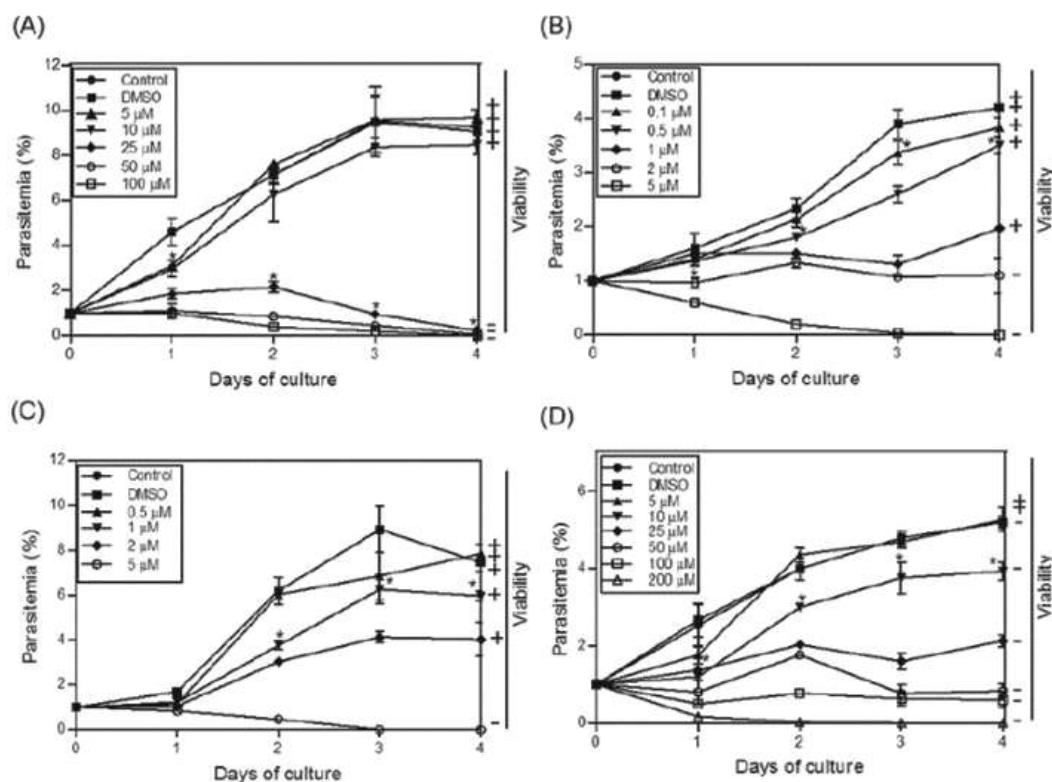[1] **Otogaru et al., 2004** ND not determined.

Fig. 1. Inhibitory effects of prumycin on the *in vitro* growth of *B. bovis* (A), *B. bigemina* (B), *B. caballi* (C), and *T. equi* (D). Each value represents the mean ± standard deviation of three separate experiments carried out in triplicate. Asterisks indicate a significant difference (Student's *t*-test; * $P < 0.05$) between the 25, 0.5, 1, and 10 µM prumycin-treated and the control cultures of *B. bovis*, *B. bigemina*, and *T. equi*, respectively.
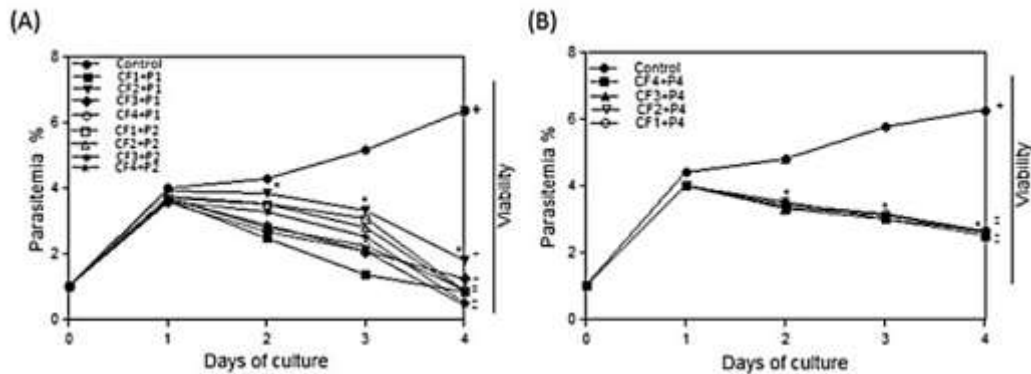
Fig. (2): Combined inhibitory effects of prumycin and clofazimine on the *in vitro* growth of *B. caballi* (A) and *B. bovis* (B). Every value shows the mean ± standard deviation of an experiment performed twice. Asterisks indicate a significant difference (Student's *t*-test;* *P* < 0.05) between the clofazimine/ prumycin-combined treatment and the control cultures.

for *B. bovis* (accession No.: XM_001610537), *B. bigemina* (accession No.: XM_012912281), and *T. equi* (accession No.: XM_004833634). Therefore, the effect of prumycin may be due to inhibition of the methionyl-tRNA-synthetase enzyme which required more research to understand the mechanism.

In conclusion, prumycin inhibited the *in vitro* growth of three *Babesia* species and *T. equi* and drug combination test of *B. caballi* and *B. bovis*. The present study indicated that prumycin might be used in combination therapy for babesiosis and theileriosis after suitable *in vivo* evaluation.

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الملخص العربي

التقييم المعملي للتأثير المثبط للبريومايسين علي نمو طفيليات البابيزيا و الثيليريا

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البريومايسين مضاد حيوي ذو طبيعه كربوهيدراتيه. تم عزله من عترة إستربتومايسيز بمحافظة كاجاوا باليابان. يمنع البريومايسين تكوين البروتين في البكتريا. يتميز البريومايسين بأنشطه مضاده للفطريات و السرطان و الملاريا. تم معمليا إختبار التأثير المثبط للبريومايسين على البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا كابالي و الثيليريا إكوي. كما تم بالإضافه لذلك أختبار التاثير المعملي المثبط لخليط البريومايسين مع الكلافوزمايين على البابيزيا بوفيزو البابيزيا كابالي. أظهرت النتائج ان الجرعه القاتله ل50% من الطفيليات كانت 22.3 و0.96 و1.89 و 21.17 ميكرومولار لكل من البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا كابالي و الثيليريا إكوي على الترتيب. كان لإضافة الكلافوزمايين للبريومايسين تأثير تآزري على طفيليات البابيزيا و الذي حسن الفاعليه على البابيزيا بوفيز والبابيزيا كابالي وقلل من جرعة البرومايسين المستخدمه في العلاج. و لذلك يمكن استخدام البريومايسين في العلاج المختلط للعدوى بالبابيزيا ولكن يلزم تجربته على الحيوانات قبل استخدامه كعلاج للحالات الإكلينيكيه.