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Original Article

In vitro inhibitory effects of puberulic acid on the growth of *Babesia* and *Theileria* parasites

Mahmoud AbouLaila[1,2*], Maram Mahmoud[3], Heba Wheeb[2], Arizoan Guswanto[1], Aki Ishiyama[4], Masato Iwatsuki[4], Kazuhiko Otoguro[4], Satoshi Ōmura[4], Naoaki Yokoyama[1], Ikuo Igarashi[1], and Soad Menshawy[2]

[1] Department of Disease Control, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan

[2]Department of Parasitology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Elbehera, Egypt

[3]Faculty of Pharmacy, Egypt-Japan University of Science and Technology, New Borg El-Arab City 21934, Alexandria, Egypt

[4]Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan.

*Corresponding author:
Dr. Mahmoud AbouLaila

E-mail: hethet2004@yahoo.com

Abstract:

Puberulic acid, a tropolone compound, has antibacterial and antimalarial activities. In this study, we investigated the *in vitro* inhibitory effects of puberulic acid on three *Babesia* species and *Theileria equi*. Puberulic acid inhibited the growth of *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi* with IC_{50} values of 1.79 ± 0.3, 1.58 ± 0.2, 2.6 ± 0.3, and 3.83 ± 0.4 µM, respectively, starting from an initial parasitemia of 1%. At 5 M, puberulic acid completely inhibited growth in *B. caballi*, 10 µM in *B. bovis*, and 25 µM in *B. bigemina* and *T. equi* cultures. Parasite growth was inhibited in the viability test at concentrations of 5 µM for *B. bigemina* and *B. caballi*, 10 µM for *B. bovis*, and 100 µM for *T. equi*. Puberulic acid had higher IC_{50} values than diminazene aceturate. At the concentrations used, the DMSO solvent did not influence the growth of the parasites. According to the *in vitro* results, puberulic acid may be a promising candidate for developing a new antibabesial drug. More research, including extensive *in vivo* assessment, is urgently needed.

Key words: Puberulic acid- *Babesia parasites- Theileria equi - In vitro*

INTRODUCTION

Babesia bovis and *Babesia bigemina* are the main causative agents of bovine babesiosis worldwide ***(Bock et al., 2008)***. *Theileria equi* and *Babesia caballi* are the major pathogens of equine piroplasmosis ***(Onyiche et al., 2019)***. The disease signs include fever, hemolytic anemia, jaundice, and hemoglobinuria. These piroplasms affect animal production and trade in many areas of the world ***(Bock et al., 2008; Onyiche et al., 2019)***. Therefore, there is an urgent need to develop either an effective vaccine or a safe drug, without side effects, to control these parasites.

Metabolic products of microorganisms are promising sources for developing new drugs for several pathogens. They had anthelmintic and insecticidal activities ***(Bentley, 2008)***. Furthermore, they had antileishmanial ***(Adler-Moore et al., 2002)***, antitrypanosomal ***(Nihei et al., 2002)***, antimalarial ***(Otoguro et al., 2003)***, and antiamoebic and antigiardial ***(Liu and Weller, 1996)*** activities. Fungi are one of these microorganisms that produce several effective compounds, such as Tropolones.

Tropolones are a type of fungal secondary

metabolite with the structural motif of an 11-membered macrocycle derived from humulene ***(Li and Cox, 2022)***. Tropolone biosynthesis in *Penicillium stipitatum* has been studied, and a gene cluster encoding the biosynthesis of the fungal tropolone stipitatic acid in *Talaromyces stipitatus*, a synonym for *P. stipitatum*, has been discovered ***(Bentley and Thiessen, 1963; Davison et al., 2012)***. Tropolone compounds, either natural or synthetic, have several activities such as antibacterial, antifungal, insecticidal, antiviral, and antitumor ***(Iwatsuki et al., 2011)***.

Puberulic acid, a tropolone compound, was first extracted from the culture broth of a *Penicillium* species ***(Birkinshaw and Raistrick, 1932; Iwatsuki et al., 2011)***. Puberulic acid was chemically synthesized ***(Sennari et al., 2014; Saito et al., 2021)***. It has antibacterial ***(Oxford et al., 1942)*** and antimalarial ***(Iwatsuki et al., 2011)*** activities. As *P. falciparum* and *Babesia* parasites share several similarities ***(Krause et al., 2007)***, the objective of this study was to determine the inhibitory effect of puberulic acid on three *Babesia* species and *Theileria equi in vitro*.

MATERIALS AND METHODS

1. Chemicals:

Puberulic acid was synthesized at the Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan. We prepared a stock solution of 10 mM in dimethyl sulfoxide (DMSO). Diminazene aceturate, Ganaseg from Cieba-Gigi, Japan Ltd., was used as a control drug. A stock solution in double-distilled water (DDW) was prepared as previously indicated ***(AbouLaila et al., 2020)***. The nucleic acid stain SYBR Green I (SGI) was obtained from Lonza, USA, at a concentration of 10,000× and stored at –20 °C to be thawed Just before use, also a lysis buffer was prepared earlier, as per the protocol described by ***Rizk et al. (2018)***, which comprised Tris (130 mM; pH 7.5), Ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V). The buffer was stored at 4 °C until use.

2. Parasites:

The parasites used in this study were *B. bovis* (Texas strain), *B. bigemina* (Argentina strain), and USDA strains of *B. caballi* and *T. equi* ***(Tuvshintulga et al., 2016)***.

3. *In vitro* culture of the parasites:

We used a continuous microaerophilous stationary phase culture system to grow parasites in cow or horse red blood cells ***(Bork et al., 2004)***. For *B. bovis*, *B. bigemina*, and *T. equi*, the culture medium M199 (Sigma-Aldrich) was supplemented with 40% cow or horse serum, 60 U/ml penicillin G, 60 mg/ml streptomycin, and 0.15 g/ml amphotericin B (Sigma-Aldrich). At 13.6 mg/ml, hypoxanthine from ICN Biomedicals, Inc. (Aurora, OH, USA) was included in the *T. equi* culture as an energetic enhancer. Antibiotics, amphotericin B, and 40% horse serum for *B. caballi* were added to the RPMI 1640 medium ***(AbouLaila et al., 2010a)***.

4. Effect of puberulic acid on host erythrocytes

Erythrocytes, bovine or equine, were incubated at 37 °C with puberulic acid at 100 µM for 3 hours and then washed with phosphate-buffered saline (PBS) three times. Subsequently, the erythrocytes were used to culture *B. bovis* and *T. equi* for 3 days using the same culture media and conditions. Fresh erythrocytes were employed as controls for culturing the two parasites. The growth of both types of cultures was examined, and any differences were recorded ***(AbouLaila et al., 2021)***.

5. *In vitro* growth inhibition assay:

Puberulic acid *in vitro* inhibition was determined using a fluorescence-based

assay, as previously described ***(Rizk et al., 2018)***. We added cow and horse RBCs with 1% parasitemia to the culture at hematocrit (HCT) values of 2.5% for *B. bovis* and *B. bigemina* and 5% for *B. caballi* and *T. equi*-packed RBC inoculum. Each well had a total volume of 100 µl. Puberulic acid concentrations of 1, 5, 10, 25, 50, and 100 µM were used. Negative controls containing no drug and either fresh or infected RBCs with the same HCT value were included. Diminazene aceturate concentrations of 5, 25, 50, 250, 1000, and 2000 nM were used. To see if the solvent had any effect on parasite growth, DMSO control plates were prepared for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* using bovine and equine RBCs at the same HCT values and puberulic acid concentrations as in the drug experiment. In a humidified multi-gas water-jacketed incubator, the plates were incubated for four days without shifting media at 37 °C in an atmosphere containing 90% N2, 5% CO2, and 5% O2. A ×2 SGI (10,000x) nucleic acid stain was mixed with 100 µl of lysis buffer and added directly to each dilution by light mixing ***(Rizk et al., 2018)***, then stored at room temperature for 6 hours in the dark. The fluorescence values at 485 nm (excitation) and 518 nm (emission) wavelengths were determined using a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific, USA). The experiments were carried out three times in triplicate. The parasitemia levels were deliberately increased after the RBC background was removed ***(Rizk et al., 2018)***. To obtain IC_{50} values, the values were used to create a regression curve.

6. Viability test:

Plates were prepared similarly to the *in vitro* inhibition assay and incubated in the same media for four days. The media were left alone, and infected erythrocytes were transferred to a new plate containing 100 µl of culture medium alone. The percentages of infected and fresh RBCs in the total RBC concentration were 42.8 and 57.2%, respectively. Plates were incubated in the same media for five days **(*AbouLaila et al., 2018*)**.

7. Statistical analysis:

Using a student's *t*-test ($P < 0.05$), JMP software (SAS Inc., USA) was used to detect significant values between different concentrations and the control.

RESULTS

Puberulic acid inhibited parasite growth significantly (student's *t*-test, $P < 0.05$) at 2 µM for *B. bovis*, *B. bigemina*, and *B. caballi* and 5 µM for *T. equi*. The IC_{50} values for *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi* were 1.79 ± 0.3, 1.58 ± 0.2, 2.6 ± 0.3, and 3.83 ± 0.4 µM, respectively (Table 1). At 5 nM diminazene aceturate treatment, *Babesia* species were significantly inhibited (Student's *t*-test, $P < 0.05$). Diminazene aceturate IC_{50} values for *B. bovis*, *B. bigemina*, *B. caballi*, and *B. equi* growth inhibition were 338 ± 20, 163 ± 18, 7.1 ± 1, and 612 ± 45 nM, respectively. Puberulic acid is more effective for bovine *Babesia* than equine piroplasms. Diminazene aceturate is particularly lethal to *B. caballi*. Puberulic acid inhibited growth at 5 µM for *B. caballi*, 10 µM for *B. bovis*, and 25 µM for *B. bigemina* and *T. equi*. Diminazene aceturate-cured parasites were fully suppressed at a concentration of 2000 nM, whereas *B. caballi* required a concentration of 50 nM to be suppressed. In the puberulic acid viability test, parasite growth was inhibited at concentrations of 5 µM for *B. bigemina* and *B. caballi*, 10 µM for *B. bovis*, and 100 µM for *T. equi* (Table 2). The diminazene aceturate-cured parasites did not re-grow in the succeeding diminazene aceturate viability test at concentrations of

Table (1): IC_{50} values of puberulic acid for *Babesia* and *Theileria* parasites

	IC_{50} values (µM)	
Parasite	**Puberulic acid**	**Diminazene aceturate**
B. bovis	1.79 ± 0.3	0.338 ± 0.020
B. bigemina	1.58 ± 0.2	0.163 ± 0.018
B. caballi	2.6 ± 0.3	0.0071 ± 0.001
T. equi	3.83 ± 0.4	0.612 ± 0.045
*P. falciparum**	0.051	ND [a]
Human MRC-5 cells*	291.72	ND [a]

[a] ND is not determined. *Iwatsuki et al., 2011

Table (2): Viability of *Babesia* species and *T. equi* after 5 days of puberulic acid treatment

	Concentration (µM)						
Parasite	**1**	**2**	**5**	**10**	**25**	**50**	**100**
B. bovis	+	+	+	—	—	—	—
B. bigemina	+	+	—	—	—	—	—
B. caballi	+	+	—	—	—	—	—
T. equi	+	+	+	+	+	+	—

(+) viable and (—) dead

25 nM for *B. caballi* and 1000 nM for *B. bovis*, *B. bigemina*, and *T. equi*. At the concentrations used, the DMSO solvent did not influence the growth of the parasites. Puberulic acid showed no effect on the host erythrocytes, and the growth of the parasites was similar to that in the control culture.

DISCUSSION

Bovine and equine piroplasmosis are tick-transmitted blood parasites. The disease signs include fever, hemolytic anemia, jaundice, and hemoglobinuria. These piroplasms affect animal production and trade in many areas of the world. An urgent search for effective drugs is needed because of the side effects of the current drugs. Puberulic acid, a tropolone compound, is produced by the *Penicillium* species. It has antibacterial ***(Oxford et al., 1942)*** and antimalarial ***(Iwatsuki et al., 2011)*** activities. Due to the similarity of *P. falciparum* and *Babesia* parasites, we were encouraged to examine its *in vitro* effects on the growth of the *Babesia* species and *T. equi*.

The growth of the four parasites was inhibited by the treatment at micromolar concentrations ranging from 1.79 to 3.83 µM. The solvent DMSO did not alter the growth of the parasites at the doses used. *B. bigemina* and *B. bovis* were more susceptible than *B. caballi* and *T. equi*. The IC_{50}s of puberulic acid were higher than those of diminazene aceturate reported in this study.

IC_{50} values of puberulic acid were higher than tulathromycin ***(Silva et al., 2018)***, actinonin on *Babesia* species ***(AbouLaila et al., 2014)***, epoxomicin ***(Aboulaila et al., 2010a)***, ***luteolin (AbouLaila et al.,***

2019a), enrofloxacin ***(AbouLaila et al., 2019b)***, quercetin ***(AbouLaila et al., 2019c)***, clofazimine for *T. equi* ***(Tuvshintulga et al., 2016)***, Coumermycin A1 ***(AbouLaila et al., 2021)***, and methylene blue ***(Tuvshintulga et al., 2015)***. While the IC_{50}s of puberulic acid were lower than thymoquinone ***(El-Sayed et al., 2019)***, resveratrol ***(El-Sayed et al., 2023)***, enoxacin ***(Omar et al., 2016)***, apigenin except for *T. equi* ***(AbouLaila et al., 2018)***, gallic acid ***(AbouLaila et al., 2018)***, trifluralin derivatives ***(Silva et al., 2013)***, (-)-Epigallocatechin-3-gallate except for *T. equi* ***(Aboulaila et al., 2010b; AbouLaila et al., 2011)***, gedunin ***(Azirwan et al., 2013)***, ciprofloxacin, thiostrepton, and clindamycin on bovine *Babesia* and *T. equi* ***(Aboulaila et al., 2012)***, cryptolepine hydrate ***(Batiha et al., 2020)***, atranorin ***(Beshbishy et al., 2020)***, clofazimine on *Babesia* species ***(Tuvshintulga et al., 2016)*** and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) ***(Guswanto et al., 2018)***.

IC_{50}s of puberulic acid (1.79–3.83 µM) are higher than that of the IC_{50} of *P. falciparum* of 0.051 µM (0.01 µg/ml) ***(Iwatsuki et al., 2011)***. This may be related to differences in parasite type and culture conditions. Puberulic acid IC_{50} values are very low compared with the IC_{50} value of 291.7 µM (57.2 µg/ml) for human MRC-5 cells ***(Iwatsuki et al., 2011)***. Therefore, it might be safe for the treatment of piroplasmosis.

The mechanism of action of puberulic acid on *Babesia* and *Theileria* parasites is not yet known. Puberulic acid has been shown to inhibit gram-positive bacteria ***(Oxford et al., 1942)*** and malaria ***(Iwatsuki et al., 2011)***. In general, both natural and synthetic tropolones exhibited a wide range of biological activities, including antibacterial, antifungal, insecticidal, antiviral, and antitumor properties. Furthermore, they have been found to inhibit various enzymes, such as aminoglycoside-2-O-adenyltransferase ***(Allen et al., 1982)***, metalloprotease ***(Morita et al., 2003)***, and HIV-1 reverse transcriptase-associated ribonuclease H ***(Budihas et al., 2005)***. Also, tropolones' inhibitory mechanisms were thought to reflect their ability to form complexes with divalent cations ***(Bentley, 2008)***. There are several ribonucleases found in the *B. bovis* genome ***(Yamagishi et al., 2014)***, such as ribonuclease H (GenBank accession No.: BBOV_I000890), CAF1 family ribonuclease containing protein (GenBank accession No.: BAN65002), ribonuclease P/MRP family protein subunit (GenBank accession No.: XP_051623651), and RNase H2 complex component family protein (GenBank accession No.: XP_001611364). Furthermore, several ribonucleases are found in the *T. equi* genome ***(Kappmeyer et al., 2012)*** such as the CAF1 family ribonuclease domain-containing protein (GenBank accession No.: XP_004830989), CAF1 family ribonuclease domain-containing protein (GenBank accession No.: AFZ81323) and ribonuclease H1 large subunit (GenBank accession No.: XP_004830217 and AFZ80551). Moreover, there are some metalloproteases in the *B. bovis* genome such as Aminopeptidase I zinc metalloprotease (GenBank accession No.: BBOV_IV011550) and ATP-dependent metalloprotease (GenBank accession No.: BBOV_III005230). Also, in the *T. equi* genome such as metalloprotease/cell

division cycle (GenBank accession No.: XM_004833698); therefore, the effect of puberulic acid might be due to targeting one or more of these enzymes, but this hypothesis requires further investigation.

CONCLUSION

According to the *in vitro* results, puberulic acid may be a promising candidate for the development of a new antibabesial drug. More research, including extensive *in vivo* assessment, is urgently needed.

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الملخص العربي

مدى تأثير حمض البيوبيروليك على نمو طفيليات البابيزيا و الثيليريا إكوي مختبريا

محمود أبوليلة [1] ومرام محمود[3] وهبه وهيب واريزوان جيسوانتو[1] واكي ايشييياما[4] وماساتو ايواتسوكي[4] وكازوهيكو اوتوجارو[4] وساتوشي ايميورا[4] وناواكي يوكوياما[1] وإكيوإجراشي وسعاد منشاوي[2]

قسم السيطره على الأمراض-المركزالقومي لأمراض البروتوزوا جامعة أوبيهيرو-اليابان [1] ، قسم الطفيليات كلية الطب البيطري جامعة دمنهور-مصر[2] و[3] كلية الصيدله- الجامعة المصريه اليابانيه-مدينة برج العرب-مصر

حمض البيوبيروليك، هو مركب تروبولون، لـه نشـاطات مضـادة للبكتيريـا والملاريـا. فـي هـذه الدراسـة، قمنـا بالتحقيق في التأثيرات المثبطة لحمض البيوبيروليك على ثلاثة أنواع من البابيزيا والثايليريا إكواي في المختبر. ثبط حمض البيوبيروليك نمو البابيزيا بوفيز، والبابيزيا بيجيمينا، والبابيزيا كابالي، والثيليريا إكـواي بقـيم IC_{50} بلغت 1.79 ± 0.3، 1.58 ± 0.2، 2.6 ± 0.3، و3.83 ± 0.4 ميكرومولار على التـوالي، بـدءًا مـن طفيليـة أولية بنسبة 1٪. عند 5 ميكرومولار، ثبط حمض البيوبيروليك النمو تمامًا في طفيليـات البابيزيـا كابـالي، و10 ميكرومـولار فـي البابيزيـا بـوفيز، و25 ميكرومـولار فـي البابيزيـا بيجيمينـا والثيليريـا إكـواي. تـم تثبـيط نمـو الطفيليـات فـي اختبـار إعـادة الحيويـة بتركيـزات 5 ميكرومـولار للبابيزيـا بيجيمينـا والبابيزيـا كابـالي، و10 ميكرومولار للبابيزيا بوفيز، و100 ميكرومولار للثايليريا إكواي. كان لحمض البيوبيروليك قيم IC_{50} أعلى مـن أسيتات الديمينازين. في التركيزات المستخدمة، لم يؤثر المذيب دايمثيـل سلفوكسـيد علـى نمـو الطفيليـات. وفقًـا للنتائج المختبرية، قد يكون حمض البيوبيروليك مرشحًا واعدًا لتطوير عقار جديد مضاد للبابيزيا. هنـاك حاجـة ملحة لمزيد من البحث، بما في ذلك تقييم واسع النطاق في الكائنات الحية.