GROWTH INHIBITORY EFFECT OF TRICLOSAN ON BOVINE AND BOvine BABESIA PARASITES

SABINE BORK, NAOAKI YOKOYAMA, TOMOHIDE MATSUO, FLORENCIA G. CLAVERIA, KOZO FUJISAKI, AND IKUO IGARASHI
National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan; Biology Department, College of Science, De La Salle University-Manila, Manila, The Philippines

Abstract. We evaluated the growth inhibitory effect of triclosan, which has recently been reported to inhibit the growth of Plasmodium species and Toxoplasma gondii, on bovine and equine Babesia parasites in in vitro cultures. The growth of Babesia bovis and B. bigemina was significantly inhibited in the presence of 100 μg/ml of triclosan, while B. caballi and B. equi were completely cleared as early as on the first and second day of the treatment, respectively. These parasites did not exhibit any growth in the subsequent five-day period of subculture without triclosan. Drug-treated parasites appeared pyeotic and atypically shaped, and ultrastructurally showed pronounced vacuolations, leading to complete destruction of parasites. Light microscopy showed that used concentrations of triclosan showed no toxicity against the host cells. The results suggest that triclosan can be used for chemotherapy of babesiosis.

INTRODUCTION

The babesias are tick-transmitted protozoans that comprise some of the most ubiquitous and widespread parasites of erythrocytes in a wide range of wild and economically valuable domestic animals such as cattle and horses, and also in humans. Clinical manifestations of babesiosis include malaria, fever, hemolytic anemia, hemoglobinuria, and edema. Thus, from the economic and public health perspectives, sustained research on babesiosis and the continued search for effective chemotherapeutic drugs are important, as is the development of practical control strategies. Several babesicidal drugs that have been in use for years have proved to be ineffective owing to problems related to their toxicity and the development of parasite resistance to these drugs. Although the problem of drug resistance in the Babesia species has not reached as high a level of concern as that reported for malarial parasites worldwide, the indiscriminate use of anti-Babesia prophylactic agents, including the administration of the drug at sublethal blood levels to animals, could actually contribute to the development of drug-resistant strains.

Triclosan, a member of a class of synthetic 2-hydroxydiphenyl ethers, exhibits broad-spectrum antimicrobial activity and is widely used as a component of deodorant soaps, dermatologic and topical skin preparations, oral rinses, and toothpastes, among others. The growth inhibitory effect of triclosan on Plasmodium falciparum and Toxoplasma gondii in vitro and rodent P. berghei in vivo has been recently reported. Because of the close biologic similarities of malarial and Toxoplasma parasites and babesias, we were encouraged to evaluate the efficacy of triclosan against the bovine and equine Babesia species. Herein, we report our findings on the inhibition of the growth of Babesia bovis, B. bigemina, B. equi, and B. caballi in an in vitro culture in the presence of triclosan.

MATERIALS AND METHODS

In vitro cultivation of Babesia parasites. Triclosan was evaluated for its chemotherapeutic effect against B. bovis (Texas strain), B. bigemina (Argentina strain), and the U.S. Department of Agriculture strains of B. equi and B. caballi. Parasites were grown in bovine and equine red blood cells using a previously established continuous micro-aerophilous stationary phase culture system. The red blood cells were collected following the method of Vega and others with some modifications as described below. Venous blood was extracted from the jugular veins of adult cows and horses and defibrinated by shaking with glass beads for 30 min. Theuffy coat was completely removed to exclude leukocytic contamination. The erythrocytes were then washed three times in phosphate-buffered saline by centrifugation at 910 × g for seven minutes at 4°C. Vega and Martinez solution (108.8 μM CaCl₂, 5.36 mM KCl, 0.4 mM KH₂PO₄, 624 μM MgSO₄, 4.04 mM Na₂HPO₄, 131.89 mM NaCl, 0.25 mM adenine, 0.5 mM guanosine, and 113.7 mM dextrose) was then added to the red blood cells at a ratio of 1.5:1.0. The erythrocytes were then stored at 4°C, and sera were stored frozen at −30°C until use. Two culture media, M199 (applied to the bovine Babesia spp. and B. equi) and RPMI 1640 medium for B. caballi (both obtained from Sigma-Aldrich, Tokyo, Japan), supplemented with 40% bovine or horse sera and 60 U/ml of penicillin G, 60 μg/ml of streptomycin, and 0.15 μg/ml of amphotericin B (Sigma-Aldrich) were added to the cultures. Hypoxanthine (13.61 μg/ml) (ICN Biomedicals, Irvine, CA) was added to the B. equi cultures as a vital supplement, while TES-hemisodium salt (229 mg/ml) (N-tris-(hydroxymethyl)-methyl-2-aminoethansulfonic acid; 2-[2-hydroxy-1,1-bis-[hydroxymethyl]ethyl]amino)ethanesulfonic acid; Sigma-Aldrich) was added to bovine Babesia parasite cultures as a pH stabilizer (pH 7.2).

Drug and dimethyl sulfoxide (DMSO) toxicity tests. Triclosan [5-chloro-2-(2,4 dichlorophenoxy)-phenol] (Sigma-Aldrich) was used as the test drug. Stock solutions of 100 and 500 mg/ml of triclosan in DMSO (Wako, Osaka, Japan) were prepared and stored at −30°C. Triclosan was dissolved in 0.005% DMSO at the highest concentration applied to the in vitro cultured parasites. As the preliminary test, the possible toxicity was examined at DMSO concentrations of 0.005% and 0.5% (as a 100 times higher concentrated control) in the species-specific growth medium, and these media were applied to the parasite cultures in triplicate. Three wells received DMSO-free growth medium as a control. This experiment was carried out three times. The range of drug concentrations selected for use in the experiments was based on the results of earlier preliminary tests (B. bovis and B. bigemina were tested at 0.05–500 μg/ml and B. equi and B. caballi were tested at 0.02–200 μg/ml).
Growth inhibition assay and viability test. The *in vitro* growth inhibitory assay was adopted from that of Igarashi and others.\(^{18}\) Bovine and equine *Babesia* parasites were obtained from cultures with parasitemias of 3.5−6.0% and 7.0−10%, respectively. All parasite cultures for drug evaluation were adjusted to 1% or 10% initial parasitemia. The growth inhibitory assay was performed in 24-well plates. One hundred microliters of the parasite bovine or equine red blood cell mixture was dispensed per well together with 1.0 mL of the culture medium containing the indicated drug concentration. Concentrations of 500, 100, and 20 \(\mu\)g/ml of triclosan were tested against the bovine parasites, while 200, 50, and 10 \(\mu\)g/ml were tested against the equine parasites. Triclosan was also added at a concentration of 4 \(\mu\)g/ml to a culture of *B. bigemina*, and at a concentration of 2 \(\mu\)g/ml to a culture of *B. caballi*. For the control, similar cultures were prepared without the drug. The experiments were carried out in triplicate per drug concentration/parasite species and in three separate trials. Cultures of *B. bovis*, *B. bigemina*, and *B. equi* were incubated at 37°C in an atmosphere of 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\), while *B. caballi* was incubated in an atmosphere of 5% CO\(_2\). For a period of four (cultures with an initial parasitemia of 1%) or three (cultures with 10% initial parasitemia) days, the culture medium was replaced every day with 1.0 mL of fresh medium containing the appropriate drug concentration. Parasitemia was monitored on the basis of approximately 1,000 erythrocytes in a Giemsa-stained thin erythrocyte smear. After four (cultures with an initial parasitemia of 1%) or three (cultures with an initial parasitemia of 10%) days of the treatment, 70 \(\mu\)L of parasite-free equine or bovine red blood cells was added to 30 \(\mu\)L of the previously drug-treated cultures in 1.0 mL of fresh growth medium without the drug. The fresh growth medium was replaced every day for the next five days, and parasite recrudescence was determined after removal of the drugs.

**Morphologic studies.** Changes in the morphology of treated *Babesia* parasites were compared with the control using light and electron microscopy. For the transmission electron microscopy, the standard Araldite embedding method was applied. The infected erythrocytes were fixed with 5.0% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at 4°C for 12 hours and washed four times with the same buffer. The specimen were embedded in 3.5% low-melting agar, postfixed with 2.0% OsO\(_4\) in the same buffer, washed again three times, and...
and dehydrated with an acetone series. The samples were then embedded in pure araldite and polymerized at 45°C and 70°C. Subsequently, ultra-thin sections (approximately 70 nm) were cut on a Leica (Wetzlar, Germany) UCT ultramicrotome using a diamond knife and double-stained with 4% uranyl acetate in 50% ethanol and lead citrate following the method of Reynolds19 before observation in a Hitachi (Tokyo, Japan) H-7500 electron microscope.

**Statistical analysis.** Differences in percent parasitemia were statistically analyzed using the independent Student’s t test at \( P < 0.01 \) as the value representing significant difference.

**RESULTS**

**Effect of DMSO on the parasites and the host cells.** To ascertain the non-toxicity of the DMSO concentrations used as drug solvent for triclosan, we exposed the parasites to 0.005% and 0.5% DMSO in species-specific growth media (Figure 1). A concentration of 0.005% DMSO did not influence the growth of the parasites as determined by light microscopy, and may therefore be considered non-toxic to the parasites. In contrast, the 100 times higher concentration (0.5%) of DMSO slightly suppressed the growth of *B. bigemina* (Figure 1b) and of the equine *Babesia* spp. (Figure 1c and d), but not of *B. bovis* (Figure 1a). Furthermore, the morphology of the host cells exposed to DMSO was not altered.

**Growth inhibitory effects in vitro.** The growth of *B. bovis* (Figure 2a) and *B. bigemina* (Figure 2c) from an initial parasitemia of 1% was significantly suppressed in the presence of 100 and 500 μg/ml of triclosan, while a drug dose as low as 50 μg/ml inhibited the growth of *B. caballi* (Figure 3a) and *B. equi* (Figure 3c). The 50% inhibitory concentration (IC\textsubscript{50}) values for triclosan on the final day of cultivation were 60 μg/ml for *B. bovis*, 54 μg/ml for *B. bigemina*, 29 μg/ml for *B. equi*, and 4.8 μg/ml for *B. caballi*. Subsequent cultivation of the parasites without the drug for a five-day period showed no regrowth of the parasites at the following drug concentrations: 100 and 500 μg/ml for the bovine parasites (Figure 2a and c), 200 μg/ml for *B. equi* (Figure 3c), and 50 and 200
µg/ml for *B. caballi* (Figure 3a). Parasites exposed to lower drug concentrations started to grow again, as shown by light microscopy. Individual parasite species exhibited differences in their susceptibility to triclosan, but these differences were not statistically significant. Complete clearance of *B. bigemina* (Figure 2c) and *B. caballi* (Figure 3a) was observed as early as the first and second days of drug treatment, respectively. The pattern of parasite growth inhibition by triclosan in cultures with an initial parasitemia of 10% (Figures 2b and d and 3b and d) was similar to that of cultures with an initial parasitemia of 1% (Figures 2a and c and 3a and c).

**Morphologic changes of parasites by the drug treatment.** To study the morphologic changes of the parasites caused by triclosan, damaged parasites were examined by light and electron microscopy and compared with intact *Babesia* parasites. At the light microscopic level, the typical parasitic shapes disappeared and pycnosis occurred in all triclosan-treated cultures. All stages of the babesia were affected by triclosan. Based on light microscopic observations of the changes in the host cell shape, size, and color, and the appearance of perforations, triclosan was non-toxic to erythrocytes.

In the triclosan-treated bovine *Babesia* parasites, we observed some damaged merozoites at an obtuse angle (*B. bovis*, Figure 4b) and at an acute angle (*B. bigemina*, Figure 4d), as well as damaged trophozoites. As shown in Figure 4f, treatment with triclosan resulted in the destruction of trophozoites of *B. caballi*. Triclosan also caused the destruction of tetrad forms of *B. equi*, as well as trophozoites in multiply infected erythrocytes (Figure 4h).

The effects of drug treatment on the ultrastructure of *B. bovis* and *B. equi* were studied using transmission electron microscopy. The fine structure of apical complexes in untreated control parasites of *B. bovis* (Figure 5a) and the intracellular organelles of *B. equi* (Figure 6a) were apparent. Drug-treated *Babesia* were irregularly shaped and showed pronounced organelle damage, including marked formation of large vacuoles (Figures 5b, c, and d and 6b and c). Conversely, untreated parasites appeared to be intact and had a pear-shaped form in *B. bovis* (Figure 5a) or a spherical form in *B. equi* (Figure 6a).

**FIGURE 3.** Growth rates of *Babesia caballi* (a and b) and *B. equi* (c and d) in the presence of various concentrations of triclosan in cultures with initial parasitemias of 1% (a and c) and 10% (b and d). Each value is the mean ± SD of three separate trials in triplicate. Parasite viability was monitored in subcultures for five days in the absence of the drug. + = viable; − = dead. Asterisks indicate a significant difference (*P* < 0.01) between the treated groups and the control group. Data are representative of three separate experiments.
DISCUSSION

Higher concentrations of triclosan inhibited the growth of both bovine and equine babesias, prevented parasite re-growth in subsequent subcultures, and caused the parasites to degenerate. However, no toxic effects on the host erythrocytes were seen. Dimethyl sulfoxide alone at the concentration used in the triclosan tests did not affect the growth of the parasites; thus, the inhibition observed was due only to drug effects. Earlier tests showed that triclosan was effective against Plasmodium species and T. gondii in vitro with IC\textsubscript{50} values of 150 ng/ml for P. falciparum and 62 ng/ml for T. gondii,\textsuperscript{13} respectively. The IC\textsubscript{50} values in the present study ranged from 4.8 μg/ml to 60 μg/ml, possibly indicating a lower sensitivity of the tested Babesia species to triclosan. In P. falciparum, only young trophozoites, but not the ring stages and the merozoites, showed a marked susceptibility to triclosan,\textsuperscript{12} whereas in the current study, all stages of the babesia appeared to be affected by the drug. However, a direct comparison of our results to those obtained in the previous experiments\textsuperscript{12,13} is difficult due to differences in culture and/or toxicity quantitation methods.

It has been proposed that Triclosan acts on specific cellular target(s), rather than causes a nonspecific disruption of the cell membrane.\textsuperscript{20} Its growth inhibitory effect on Plasmodium and Toxoplasma species\textsuperscript{12,13,21} has been associated with its action on enoyl acyl carrier protein reductase, an enzyme used in fatty acid synthesis that is located in a plastid-like organelle.\textsuperscript{22} This organelle was also detected in Sarcocystis muris, B. ovis, and B. bovis.\textsuperscript{23–26} At the present time, we can only speculate a similar mode of drug action against bovine and equine babesias. Further studies confirming the presence of such an organelle in B. bigemina, B. equi, and B. caballi and identification of the genes encoding drug target are being pursued.

The induction of the formation of autophagic vacuoles as a type of secondary lysosome has been demonstrated in P. berghei treated with chloroquine, Trypanosoma cruzi treated with cis-diaminedichloroplatinum (II) analogs, and Entamoeba histolytica, Giardia lamblia, and Trichomonas vaginalis.

FIGURE 4. Morphologic changes in triclosan-treated Babesia parasites. Babesia bovis: control (a) and treated (b). Note the prominent, knob-like protuberances of the infected host cells in b. B. bigemina: control (c) and treated (d). B. caballi: control (e) and treated (f). B. equi: control (g) and treated (h). Note the typical Maltese cross form in g and the degenerate or pyknotic appearance of parasites in the treated groups (b, d, f, and h).

FIGURE 5. Ultrastructural changes in Babesia bovis treated with triclosan. Treated parasites show several cytoplasmic vacuolations (V) and loss of integrity of the parasite shape, including damage to organelles (b, c, and d) compared with the normal pear-shaped intact untreated parasite (a). N = nucleus; Sb = spherical body; Rh = rhoptry; Pm = plasma membrane; Nc = nucleolus; R = residue. Bars = 0.25 μm.
treated with berberine sulfate. These earlier documentations of autophagous vacuolation and similar observations in triclosan-treated babesias in the present study point suggest that vacuole formation commonly occurs in a wide range of drug-treated protozoan parasites.

In conclusion, we have clearly demonstrated the efficacy of the anti-microbial drug triclosan at concentrations as low as 50 and 100 μg/ml against equine and bovine Babesia spp., respectively, without any indication of parasite recrudescence in vitro. Additional studies on the development of parasite resistance to triclosan, elucidation of the mode of drug action, and evaluation of the chemotherapeutic effect of triclosan in vivo are clearly needed.

Received July 29, 2002. Accepted for publication November 25, 2002.

Acknowledgments: We thank Akiko Nagai, Dr. Susanne Walter, and Dr. Badgar Battsetseg for their technical support and advice. This article was presented in part at the 42nd Annual Meeting of Japanese Society of Tropical Medicine, Tokyo, September 2001 (abstract A 16), and the 133rd Meeting of Japanese Society of Veterinary Science, Tokyo, March 2002 (article C-32 VII).

Financial support: This study was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Sciences.

Authors’ addresses: Sabine Bork, Naoko Yokoyama, Tomohide Matsuo, Kozo Fujisaki, and Ikuo Igarashi, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan, Florencia G. Claveria, Biology Department, College of Science, De La Salle University-Manila, Taft Avenue, Manila, The Philippines.

Reprints requests: Ikuo Igarashi, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan, Telephone: 81-155-49-5641, Fax: 81-155-49-5643, E-mail: igarcpm@obihiro.ac.jp

REFERENCES


bigemina: *in vitro* and *in vivo* effects of curdlan sulfate on
19. Reynolds ES, 1963. The use of lead citrate at high pH as an
electron opaque stain in electron microscopy. *J Cell Biol* 17:
208–212.
tural basis and mechanism of enoyl reductase inhibition by
closan and NAD binding to enoyl-ACP reductase of *Plasmo-
dium falciparum*. *Biochem Biophys Res Commun* 283: 224–
228.
gene with homology to the small subunit ribosomal RNA gene
ribosomal RNA gene from *Babesia bovis*: nucleotide se-
quence, secondary structure of the transcript, and preliminary
25. Hackstein JH, Mackenstedt U, Mehlhorn H, Meijerink JP, Schu-
bert H, Leunissen JA, 1995. Parasitic apicomplexans harbor a
chlorophyll a-D1 complex, the potential target for therapeutic
26. Jeffries AC, Johnson AM, 1996. The growing importance of the plas-
27. Warhurst DC, Homewood CA, Baggaley VC, 1974. The chemo-
therapy of rodent malaria. XX. Autophagic vacuole formation
in *Plasmodium berghei in vitro*. *Ann Trop Med Parasitol* 48:
265–281.
cis-diaminedichloro platinum(II) analogs. *J Parasitol* 73:
272–277.
berberine sulphate on the growth and structure of *Entamoeba
histolytica*, *Giardia lamblia*, and *Trichomonas vaginalis*. *Ann