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IN VITRO GROWTH INHIBITORY EFFECTS OF COMBINED APPLICATIONS OF CLOTRIMAZOLE, KETOCONAZOLE, AND CLODINAFOP-PROPARGYL ON EQUINE *BABESIA* PARASITES

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Abstract

Recently, we reported the *in vitro* growth inhibitory effects of the imidazole derivatives, clotrimazole (CLT) and ketoconazole (KC), and the herbicide, clodinafop-propargyl (CP), against bovine and equine *Babesia* parasites. Additionally, in the bovine species, several combined applications of the CLT, KC, and CP were reported to significantly increase the inhibitory effects. In this study, similar combination tests were carried out in order to confirm possible synergistic effects in the equine species, *Babesia caballi* and *Babesia equi*. Combinations of CLT/KC, CLT/CP, and CLT/KC/CP exerted a significantly enhanced growth inhibitory efficacy in *B. caballi*, in contrast to *B. equi*, where no synergistic effects were observed in any of the combinations. Our results suggest that the combined usage of these drugs may have a high enough potential to be considered applicable in practice.

Keywords: *in vitro*, *Babesia*, clotrimazole, ketoconazole, clodinafop-propargyl

Introduction

Equine babesiosis, also known as biliary fever, is a tick-borne disease of equidae caused by two Apicomplexan parasites, *Babesia caballi* and *Babesia equi*. These intraerythrocytic protozoa occur in many regions of the world (Friedhoff *et al.*, 1990) and are considered to be an important threat for livestock (Schein, 1988). Infected animals suffer from fever, anorexia, severe anemia, and apathy (Holman *et al.*, 1998), leading to their restricted use in sports and in the meat/horse industry. The mortality rate is as high as 50% (Levine, 1985), and the surviving horses are likely to remain subclinical carriers for life. Currently, horses awaiting importation into countries considered free of the equine babesiosis (such as Japan) have to adhere to strict regulations, including serological tests (Holman *et al.*, 1998).

Although many anti-babesial drugs have been developed and applied more or less successfully for several years (Kuttler *et al.*, 1987; Singh *et al.*, 1980; Bruning, 1996), many compounds have been shown to have severe side effects or to produce drug-resistant parasites (Adams, 1980; Granwell, 1990; Upcroft, 1994; Wijaya *et al.*, 2000). With regard to these facts, further development of potent and safe drugs is highly required. We recently demonstrated the efficacy of the imidazole derivatives and anti-fungal agents, clotrimazole (CLT) and ketoconazole (KC), and the herbicide, clodinafop-propargyl (CP), when applied singly (as mono-drug) to equine *Babesia* parasites (Bork *et al.*, 2003*a*) and singly as well as in combinations to bovine *Babesia* species (Bork *et al.*, 2003*b*) by using the *in vitro* cultivation system (Igarashi *et al.*, 1998; Nagai *et al.*, 2003; Bork *et al.*, 2003*c*, 2004). Since several studies reported the usefulness of combined drug applications with the benefit of higher sensitivity and fewer adverse effects (Krause *et al.*, 2000; Wijaya *et al.*, 2000), we were encouraged to evaluate the efficacy of drug combinations against the *in vitro* growth of *B.*

equi and *B. caballi*.

Materials and methods

Parasites and* in vitro *cultivation: United States Department of Agriculture (USDA) strains of *B. caballi* and *B. equi*, maintained in the NRCPD for several years, were grown in equine erythrocytes as described previously (Bork *et al.*, 2003*c*).

Chemicals: Clotrimazole (CLT) (1-[(2-chlorophenyl) diphenylmethyl]-1*H*-imidazole) and ketoconazole (KC) (cis-1-acetyl-4-[4-[[2- (2,4-dichlorophenyl) -2- (1*H*-imidazole-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine) were obtained from Sigma Chemical Co. (St. Louis, Missouri, U S A). Clodinafop-propargyl (CP) ((2*R*)-2-[4-[(5-Chloro-3-fluoro-2-pyridinyl) oxy]phenoxy] propanoic acid-2-propynyl ester) was purchased from Riedel-de Haën Laborchemikalien (Seelze, Germany). The stock solutions were prepared as described previously (Bork *et al.*, 2003*a*).

Drug combination tests: The concentrations of CLT, KC, and CP, acting suppressive but not destructive to the parasites, were determined according to previous experiments (Bork *et al.*, 2003*b*) and applied in the present study. The classification into "synergistic," "antagonistic," or "no effect" was made as follows: The inh.% value of the drug combination was compared to that of the drug applied singly. When the inh.% value of the combination was higher than that of the most effective singly applied drug, the result was considered to be "synergistic." In the case that the inh.% value of the drug combination was lower, it was determined to be "antagonistic." Finally, when both inh.% values (drug combination and singly applied) were almost identical, it was determined to have "no effect."

Results

Table 1 shows the percentage of growth inhibition (inh.%) of the combined applications of CLT, KC, and CP against equine *Babesia* parasites in comparison to the control inh.% of the mono-drug applications. The inh.% varied from 1.6% in *B. caballi* exposed to KC/CP to 96.2% in the same parasite exposed to the triple combination of CLT/KC/CP. In addition, in *B. caballi*, the CLT/KC, CLT/CP, and CLT/KC/CP were found to act synergistically and reveal a stronger enhancing inh.% compared to that of the single application. Only the combination of KC/CP had a significantly attenuated effect. On the other hand, in *B. equi*, the inh.% ranged from 29.4 (KC/CP) to 92% (CLT/KC). Combinations of KC/CP and CLT/KC/CP acted antagonistically; especially, the inh.% of the KC/CP combination revealed a 0.4-fold lower sensitivity in *B. equi* than that of the KC single application.

Discussion

In this study, the applicability of drug combinations containing CLT, KC, and CP was evaluated against the *in vitro* growth of *B. equi* and *B. caballi*. In *B. caballi*, all combinations containing CLT showed strong enhancing efficacies. These findings correlate with previous reports, according to which these combinations significantly enhanced the destructive effects in *B. bovis* and *B. bigemina* (Bork *et al.*, 2003*b*), but were clearly different from the results of *B. equi*. All but two combinations (KC/CP and CLT/KC/CP) acted antagonistically for *B. equi*, whereas other combinations did not show any enhancing or weakening effects. This interesting observation is consistent with previous reports describing several differences between the two equine species in the level of drug sensitivity (Schein, 1988). It is well understood that the sensitivity of

B. equi to drugs is usually lower than that of *B. caballi* (Bork *et al.*, 2003*c*; Nagai *et al.*, 2003), which might be explained through the probably distinct generic classification of *B. equi* as *Theileria equi* (Mehlhorn *et al.*, 1998). In contrast, the occurrence of strong antagonistic effects of the KC/CP combination in both equine parasites is also consistent with the results obtained in *B. bovis*, but in complete contrast to the findings in *B. bigemina* (Bork *et al.*, 2003*b*). These findings possibly suggest a common metabolic pathway among the *Babesia* parasites except *B. bigemina.*

Synergy between drugs was proved to be a valuable tool to improve treatment efficacy in several diseases (Radloff *et al.*, 1996). In a previous report, CLT exerted synergistic effects when combined with the antibiotic mefloquine, but it was found to be antagonistic with the widely used antibiotic chloroquine against *Plasmodium* parasites (Tiffert *et al.*, 2000). Combinations of KC with either a lactone derivative (lovastatin, imidazole acetamide, or benznidazole) or an ergosterol biosynthesis inhibitor (terbinafine) were found to be highly successful against *Trypanosoma cruzi* infection (Araujo *et al.*, 2000; Urbina *et al.*, 1993). In *P. falciparum*, the CLT may interfere with the hemobiosynthesis (Huy *et al.*, 2002), and, in fungi, the KC is considered to act on the microsomal P450-dependent enzyme system (Matthew *et al.*, 1993), while the CP inhibits one key enzyme in the *de novo* fatty acid biosynthesis of plants (Zuther *et al.*, 1999). Although *Plasmodium* and *Babesia* species are known to have close biological similarities (Clark and Jacobson, 1998), the action-mode of the three compounds may differ among *Babesia* parasites and, therefore, requires further clarification.

In conclusion, the usefulness of combined applications of CLT, KC, and CP was clearly demonstrated against the *in vitro* growth of *B. equi* and *B. caballi*.

Table 1. Growth inhibitory efficacy (inh.%) at suppressive concentrations of CLT, KC, and CP to the *in vitro* growth of *B. caballi* and *B. equi*

Drugs	*Babesia caballi*			*Babesia equi*		
	drug conc.	inh.%*	effects$	drug conc.	inh.%*	effects$
CLT	15μM	50.5%		2.5μM	71.4%	
KC	10μM	18.2%		10μM	93.6%	
CP	250μM	14.5%		500μM	57.0%	
CLT/KC	15μM/10μM	85.4%	syn	2.5μM/10μM	92.0%	ne
CLT/CP	15μM/250μM	92.0%	syn	2.5μM/500μM	74.0%	ne
KC/CP	10μM/250μM	1. 6%	ant	10μM/500μM	29.4%	ant
CLT/KC/CP	15μM/10μM/250μM	96.2%	syn	2.5μM/10μM/500μM	70.7%	ant

* inh.% indicates parasitemia/control parasitemia without drug addition.
$ synergistic (syn); antagonistic (ant); no effect (ne)

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