Effects of protein kinase inhibitors on the *in vitro* growth of *Babesia bovis*

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SUMMARY

Staurosporine, Ro-31-7549, and KN-93, which are inhibitors of serine/threonine protein kinase, protein kinase C, and calcium-modulin kinase, respectively, were tested for their effects on the *in vitro* growth of *Babesia bovis*. Staurosporine was the most effective inhibitor, completely clearing the parasitaemia as early as the first day of exposure at a concentration of 100 μM. Moreover, staurosporine caused a significant increase in the percentage of extracellular merozoites, most likely due to the inhibition of erythrocyte invasion by the parasite. Although 5 mM Ro-31-7549 and KN-93 had a suppressive action, this was not enough to destroy the parasite. Interestingly, concentrations of 0·5 to 5 mM KN-93 influenced the parasitic development within the infected erythrocytes. The present study suggests that *B. bovis* requires, to a certain extent, the phosphorylations mediated by parasite- or host erythrocyte-protein kinases, in particular, for the processes of successful invasion of erythrocytes and intraerythrocytic development.

Key words: staurosporine, Ro-31-7549, KN-93, *Babesia bovis*, *in vitro* cultivation.

INTRODUCTION

An intraerythrocytic protozoon, *Babesia bovis*, is one of the major causative agents of babesiosis in cattle (Sam-Yellowe, 1996; Homer *et al*. 2000). This parasite causes severe symptoms such as fever, anaemia, and cerebral dysfunctions during its erythrocytic stage, and thereby has a significant impact in the cattle industry worldwide (Kuttler, 1988). The host-parasite interaction between *B. bovis* and the host is complex. A better understanding of the biological processes involved in the growth cycle at the erythrocytic stage is required in order to develop effective therapeutic tools against bovine babesiosis (Bork *et al*. 2004*a*).

Protein kinases play essential roles in various signalling pathways of eukaryotic cells by catalysing the transfer of phosphate from ATP to an amino acid side-chain of a protein (Karin, 1991). They are vital for cell growth and survival (Bray, 1990). Any irregularity in their enzymatic activities may result in serious diseases, such as cancer, diabetes, and cardiovascular disorders (Komuro, 2001; Fulop, Larbi and Douziech, 2003; Russello and Shore, 2004). Since such protein phosphorylations are also essential in protozoa, notable advances have been made in determining the growth inhibitory activities of specific kinase inhibitors and clarifying the structure and function of the target protein kinases (Wiser and Schweiger, 1985; Flawia, Tellez-Inon and Torres, 1997; Doerig, 2004).

A serine/threonine kinase inhibitor, staurosporine (Becker and Jaffe, 1997), has broad biological activity ranging from anti-fungal (Sancelme, Fabre and Prudhomme, 1994) to anti-hypertensive (Berg, 2003). Additionally, it inhibits host cell invasion or growth of several *Plasmodium* species (Ward *et al*. 1994; Dluzewski and Garcia, 1996; Gazarini and Garcia, 2003), the epimastigotes of *Trypanosoma cruzi* (Vieira, de Carvalho and de Souza, 1994; Malaquias and Oliveira, 1999), and the promastigotes of *Leishmania major* and *L. amazonensis* (Becker and Jaffe, 1997). A selective protein kinase C (PKC) inhibitor, Ro-31-7549 (Hashimoto *et al*. 1997), is a potent down-regulator of several PKC-subtypes in human cancer cells (Turner *et al*. 1996), but study of its effect on protozoa is still lacking. Finally, a Ca^{2+}/calmodulin (CaM)-dependent protein (calcium-modulin) kinase inhibitor, KN-93 (Sumi *et al*. 1991), inhibited the growth of the epimastigotes of *T. cruzi* (Malaquias and Oliveira, 1999) and hampered the morphological development of the *P. gallinaceum* zygote to ookinete in mosquitoes (Silva-Neto, Atella and Shahabuddin, 2002). In the present work, we investigated the inhibitory activities of these protein kinase inhibitors on the growth of *B. bovis in vitro*.

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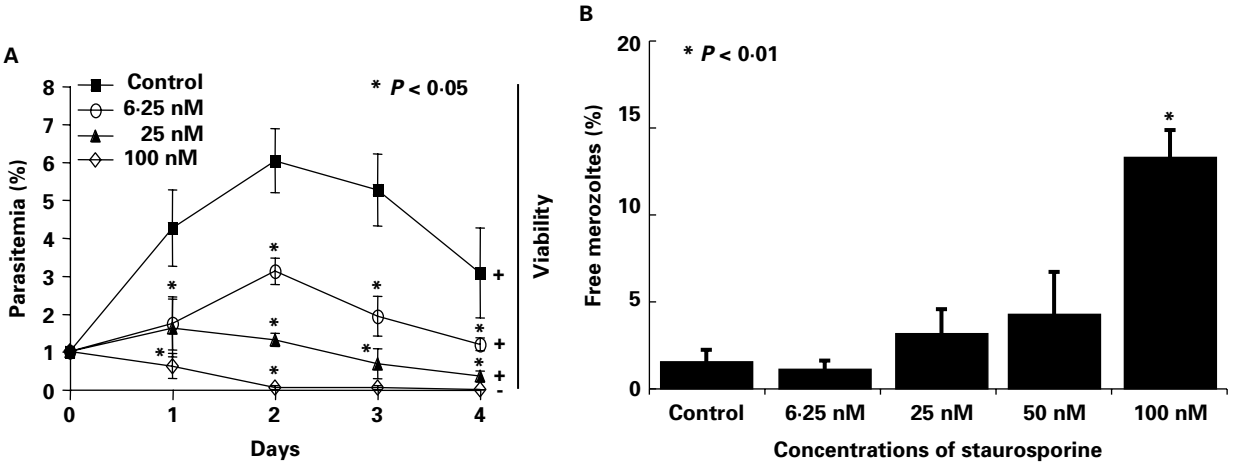

Fig. 1. (A) *In vitro* growth curves of *Babesia bovis* in the presence of different concentrations of staurosporine, based on the dynamics of parasitaemia. Parasitic viability: viable (+), dead (–). (B) Percentage of free merozoites in *in vitro* cultures of *B. bovis* exposed to different concentrations of staurosporine on the first day of culture. Each value represents the mean ± standard deviation (S.D.) in 3 separate trials carried out in triplicate. Asterisks indicate significant differences ($P<0{\cdot}05$ in (A) and $P<0{\cdot}01$ in (B)) between the drug-treated and control groups.

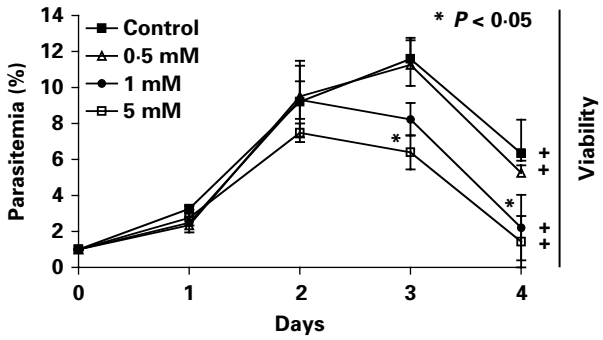

Fig. 2. *In vitro* growth curves of *Babesia bovis* in the presence of different concentrations of Ro-31-7549. Parasitic viability: viable (+), dead (–). Asterisks indicate significant differences ($P<0{\cdot}05$) between the drug-treated and control groups. Each value represents the mean ± S.D. in 3 separate trials conducted in triplicate.

MATERIALS AND METHODS

In vitro *cultivation of* B. bovis

The Texas strain of *B. bovis* was maintained in purified bovine erythrocytes in a serum-free GIT growth medium (Wako Pure Chemical Industrial Ltd., Osaka, Japan) as described previously (Bork *et al.* 2003*a*, 2004*b*, 2005).

Chemicals

Indolcarbazole staurosporine was purchased from Wako, and *bis*indolylmaleimide-VIII-acetate, Ro-31-7549, and the methoxybenzenesulfonamide KN-93 were obtained from Calbiochem (Darmstadt, Germany). Stock solutions of 1 M of the chemicals were prepared in dimethylsulfoxide (DMSO; Wako) and stored at −30 °C until use. Different drug concentrations were prepared by diluting the stock solution with the GIT medium. The concentrations of DMSO used in this study did not exert any abnormally inhibitory effects on the growth, as described previously (Bork *et al.* 2003*a*).

Growth inhibition assays

In vitro growth inhibition assays were performed as described previously (Bork *et al.* 2003*a*, *b*). In brief, 100 μl of infected bovine erythrocytes were diluted with non-infected erythrocytes to obtain 1% parasitaemia in a 0·1 ml volume, and the mixture was subsequently suspended in 0·9 ml of the growth medium supplemented with the indicated concentrations of chemicals. The suspension was added to 24-well culture plates (Nunk, Roskilde, Denmark), and the plates were incubated in a humidified multigas water-jacketed incubator at 37 °C for 4 days. During the incubation period, the overlaid culture medium was replaced daily with 0·9 ml of fresh growth medium containing the chemicals at the indicated concentrations. In parallel, chemical-free cultures were prepared as controls. Giemsa-stained thin blood smears were prepared daily, and the parasitic growth was monitored as parasitaemia by evaluating at least 1000 erythrocytes per well using light microscopy (Nikon, Tokyo, Japan). The percentage of extraerythrocytic (free) merozoites was calculated on the first day of culture as the ratio of free merozoites to the entire (intra- and extraerythrocytic) parasite population in approximately 250 parasites. The percentage of ring-shaped parasites was determined as the ratio of ring-shaped intraerythrocytic parasites to the entire amount of intraerythrocytic parasites in

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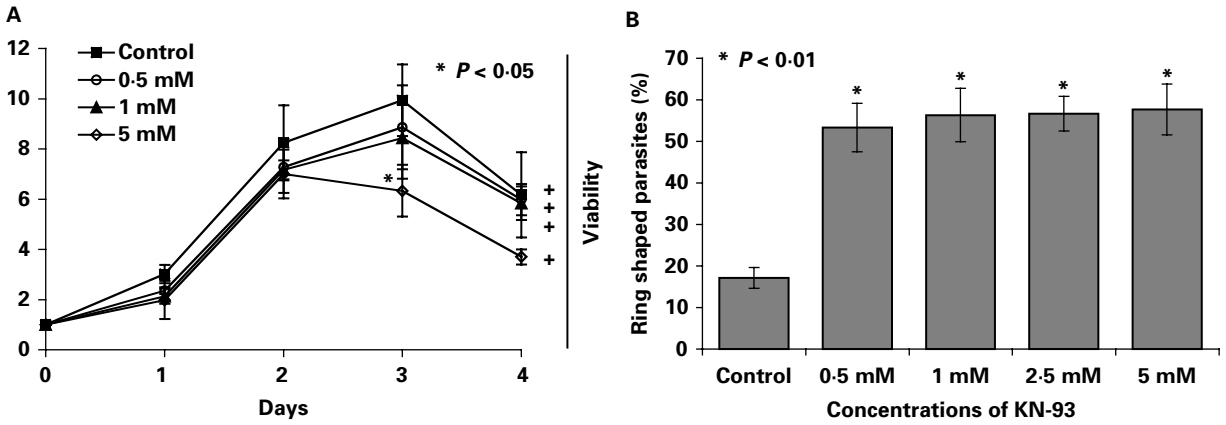

Fig. 3. (A) *In vitro* growth curves of *Babesia bovis* in the presence of different concentrations of KN-93. Parasitic viability: viable (+), dead (−). (B) Percentage of ring-shaped intraerythrocytic parasites in *in vitro* cultures of *B. bovis* exposed to different concentrations of KN-93 on the second day of culture. Each value represents the mean ± S.D. in 3 separate trials conducted in triplicate. Asterisks indicate significant differences ($P<0·05$ in (A) and $P<0·01$ in (B)) between the drug-treated and control groups.

approximately 250 parasites. Viability tests were also conducted following the method described by Bork *et al*. (2004*a*). In brief, after 4 days of chemical exposure, 30 μl of each of the infected and treated erythrocytes were mixed with 70 μl of non-infected erythrocytes and suspended in fresh growth medium without chemical supplementation. The plates were incubated for the next 10 days. The culture medium was replaced daily, and parasite recrudescence was determined by light microscopy to evaluate the parasite viability.

Statistical analysis

The 50% inhibition (IC_{50}) values of the drugs were determined in triplicate on the fourth day of *in vitro* culture after curve-fitting using Cricket Graph Software (Malvern, Pennsylvania, USA). Differences in the percentage parasitaemia and other percentages were statistically analysed using an independent Student's *t*-test at $P<0·01$ and $P<0·05$ as the values representing significant differences.

RESULTS

A concentration of 100 μM staurosporine irreversibly arrested the growth of *B. bovis* as early as the first day of *in vitro* culture, as shown in light microscopy and a subsequent viability test (Fig. 1A). During the time-course of the growth inhibition assay, concentrations from 6·25 to 50 μM significantly suppressed the parasitaemia but did not destroy the parasites. The IC_{50} value was determined as 5·12 μM. On the first day of culture, 25, 50, and 100 μM staurosporine resulted in substantial increases in the percentages of extraerythrocytic (free) merozoites, i.e. 3·2% ± 2·8, 4·8% ± 4·3, and 14·5% ± 4·1 ($P<0·01$), respectively, as compared to the non-treated control group (1·1% ± 1·1) (Fig. 1B). In the subsequent time-course with staurosporine, almost all of the free merozoites became pycnotic and died (data not shown).

Concentrations of 1 and 5 mM Ro-31-7549 reduced the parasitaemia, starting from the second and third day of culture, respectively. However, full destruction of the parasite was not observed and, additionally, a concentration below 1 mM did not exert any growth inhibitory effects on *B. bovis* (Fig. 2). The IC_{50} value was estimated as 4·83 mM.

Treatment with 5 mM KN-93 slightly but significantly hampered the parasitic growth as early as the third day of culture, but failed to completely clear the parasites from the culture (Fig. 3A). The IC_{50} value was estimated as 11·75 mM. Interestingly, on the second day of culture, the percentages of ring-shaped parasites within the infected erythrocytes increased ($P<0·01$) in the cultures treated with 0·5 mM (53·3% ± 5·8), 1 mM (56·3% ± 6·4), 2·5 mM (56·7% ± 4·2), and 5 mM (57·7% ± 6·1) of KN-93, as compared to the control (17·1% ± 2·5) (Fig. 3B).

DISCUSSION

In this study, 3 kinds of protein kinase inhibitors were shown to have substantial inhibitory effects on the *in vitro* growth of *B. bovis*. Among them, the serine/threonine kinase inhibitor, staurosporine, showed the strongest activity of growth inhibition. The significant increase in the percentage of free merozoites in the staurosporine-treated cultures might be attributed to the inhibition of the parasite's invasion into host erythrocytes. Our findings correspond to previous publications reporting that staurosporine significantly inhibited the erythrocyte invasions by *P. knowlesi* (Ward *et al*. 1994), *P. falciparum* (Dluzewski and Garcia, 1996), and *P. chabaudi* (Gazarini and Garcia, 2003) in their cultures. The inhibitory mechanism, however,

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remains unclear yet, because the significant increase of free merozoites was accomplished only at the highest concentration of staurosporine that affected the parasite viability in this study. Further study will be required to understand the inhibitory mechanism of staurosporine on *B. bovis*.

The PKC-inhibitor, Ro-31-7549, has not been studied in any protozoa. Thus, our work is the first study of its possible inhibitory effect on the propagation of *B. bovis*. Previously, Ray *et al.* (1990) demonstrated that *B. bovis* possesses a powerful PKC activity in the parasitic membrane. However, the growth inhibition activity of Ro-31-7549 was not as high as we initially expected. Whether the result in our growth inhibition assay was due to insufficient drug concentrations used in our study or the possibilities that Ro-31-7549 may not be a specific inhibitor of PKC activity in *B. bovis* or that PKC activity may not be essential for *in vitro* growth, remains to be elucidated.

Although treatment with a Ca^{2+}/CaM-dependent protein kinase inhibitor, KN-93, failed to effectively interrupt the parasitic propagation, it caused a significant increase in the percentage of ring-shaped parasites, which are the early forms after erythrocyte invasion that follow the subsequent division to 2 elongated parasites (Levine, 1988). This morphological phenomenon was also observed in the *Plasmodium* species (Silva-Neto *et al.* 2002). Ray *et al.* (1990) demonstrated the existence of a Ca^{2+}/CaM environment in *B. bovis* and mentioned a possible correlation between the growth of *B. bovis* and Ca^{2+}-dependent protein phosphorylation by kinases. For understanding the biological effect of KN-93 on *B. bovis*, it is necessary to identify the Ca^{2+}/CaM-dependent protein kinase of *B. bovis* and investigate its relationship with the morphological shape changes from ring-shaped to elongated *Babesia* parasites.

Our endeavour to work with these protein kinase inhibitors will contribute to the understanding of the biological mechanisms of erythrocyte invasion and development of *B. bovis* in future. In particular, the identification of the target protein kinases, which possibly exist in the parasite or host erythrocyte, is of major interest. A more detailed exploration of the biological roles of protein kinases involved in the asexual growth of *Babesia* parasites will pave the way for a better understanding and control of bovine babesiosis.

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