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TRYPANOCIDAL EFFECT OF SKF525A, PROADIFEN, ON DIFFERENT DEVELOPMENTAL FORMS OF *TRYPANOSOMA CRUZI* *

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Abstract SKF525A, an inhibitor and inducer of cytochrome P450, was tested on different developmental stages of *Trypanosoma cruzi*. Growth, motility and structure of epimastigotes, motility and infectivity of trypomastigotes, and infectivity of trypomastigotes to Vero cells in culture were abolished by the drug at 10-100 µM concentrations. When blood from infected mice was treated with the drug, and used to infect 8 day-old mice, no parasites were observed at 0.6-1 mM, and all animals survived. Blood cell morphology was well preserved, and the sleeping time of pentobarbital-treated mice inoculated with the same amount of drug was not increased. The present results suggest that SKF525A or other related inhibitors of cytochrome P450 coned be tested as an additive for blood sterilization in blood banks.

Resumen ***Efecto tripanocida de SKF 525A, PROADIFEN, sobre diferentes estadios del desarrollo del* Trypanosoma cruzi**. El crecimiento, la motilidad y la estructura de epimastigotes, la motilidad y la infectividad de tripomastigotes y la infectividad de tripomastigotes sobre células Vero en cultivo fueron suprimidas totalmente con concentraciones de la droga entre 10 y 100 µM. Cuando sangre de ratón infectado se trató con la droga, y luego se la utilizó para infectar ratones de 8 días de edad, no se observaron parásitos entre 0.6 y 1 mM, y todos los animales sobrevivieron. La morfología de las células sanguíneas se preservó y el tiempo de sueño de los ratones tratados con pentobarbital e inoculados con la misma cantidad de droga no se vio aumentado. Los presentes resultados sugieren que el SKF 525A u otras drogas relacionadas inhibidores del P450 podrían probarse como aditivos en la esterilización en bancos de sangre.

Key words: *Trypanosoma cruzi*, SKF525A, growth inhibition, blood bank sterilization

A large number of drugs have been tested against *Trypanosoma cruzi*, the parasitic flagellate which causes the American Trypanosomiasis, Chagas disease. Among these, there are antibiotics, amphiphilic drugs, azole derivatives, nitroheterocyclics, purine derivatives, naphthoquinones, metallic complexes, antioxidants, cysteine proteinase and tripanothione reductase inhibitors[1-4]. New drugs are necessary, not only for the treatment of chagasic patients, but for the treatment of blood to prevent transfusional transmission of the disease. The drugs available (Nifurtimox and Benznida-zole for treatment, Gentian Violet for transfusion) present a number of undesirable properties[5,6]. It is necessary, therefore, to search for new drugs, more effective and less toxic than those already available.

The aim of this study is to determine the potential trypanocidal effects of β-diethylaminoethyl-diphenylpropyl acetate hydrochloride (SKF525A or PROADIFEN). This drug interacts with the parasite's cytochrome P450 (as azole derivatives, such as ketoconazole and fluoconazole)[7-9] and shares other properties with drugs proven to be active against the parasite[3], since it has an amphiphilic structure[4, 10] and presents antioxidant properties[11].

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Materials and Methods

Obtention of parasites. Epimastigotes (Tul2 or RA strains) were grown and harvested as previously described[12]. Cell-culture trypomastigotes (RA strain) were obtained from infected Vero cells[13]. Blood samples containing bloodstream trypomastigotes (Tulahuén strain, Tul 2 stock) were obtained by cardiac puncture from infected BALB/c mice. Blood samples from inbred CF-1 male mice were used to simulate blood bank conditions

by adding cell-culture trypomastigotes, and for control determinations of the effect of SKF525A on pentobarbital sleeping time.

Assay of inhibition of motility. Epimastigotes and culture trypomastigotes (in both cases at a concentration of 5 x 10^6 parasites/ml), were suspended in fresh culture medium containing the drug concentrations stated. The suspensions were observed under the microscope, and the time for complete immobilization was recorded.

Assay of inhibition of epimastigote growth. Parasites were grown at 28° C, in the absence or in the presence of the drug concentrations stated in Fig. 1, and growth was followed by daily counting using a Neubauer chamber.

Assay of the effect of SKF525A on the parasite cycle in Vero cells. Vero cells (4.4 x 10^4/ml) were cultured at 37°C in Modified Eagle's Medium (MEM) containing 5% (v/v) fetal calf serum, in 24-well plate dishes containing glass coverslips. After 48 hr the cultures were inoculated with RA strain cell-culture trypomastigotes (3.5 x 10^5/ml), with or without preincubation (4 hr at 37°C without drug or with 10 µM SKF525A). After 24 hr the medium, containing the non-internalized parasites, was removed; fresh medium, with or without drug, was added, and the infected cells were incubated for 72 hr, and stained with May-Grünwald-Giemsa. The percentage of infected cells and the number of intracellular parasites were estimated by observing 500 cells in a Zeiss Photomicroscope II. The results are expressed as the endocytic index (product of the % of cells infected and the number of amastigotes/cell).

Assay of the effect of SKF525A on RA strain trypomastigotes suspended in mouse blood. Vero cell-derived trypomastigotes suspended in MEM containing 5% fetal calf serum were added to mouse blood, at a final concentration of 5 x 10^5 trypomastigotes/ml. Aliquots (100 µl) were sampled in microwell plates and added SKF525A at the final concentrations stated under Results. After 24 hr at 4°C, the parasites in 5 µl aliquots were counted under the microscope. Aliquots were diluted with the same medium to a final concentration of 9.0 x 10^3 trypomastigotes/ml, and used to infect Vero cell cultures. Culture medium and non-internalized parasites were removed after 24 hr, fresh medium was added, and the flasks were incubated at 37°C for up to 20 days, with periodic changes of medium. The number of released parasites was counted daily until the end of the experiment.

Assay of the effect of SKF525A on Tul 2 strain bloodstream trypomastigotes in mouse blood. The incubation of the parasites was performed as in the previous experiment, but the samples, after counting the trypomastigotes under the microscope, were inoculated into 8-days old mice. Groups of 5 male BALB/c mice from the same litter were used for each drug concentration. Parasitemia (determined in blood taken from the tail) and survival were followed up to 30 days. The animals surviving after 30 days were sacrificed, and homogenates of heart or liver, and blood, were inoculated into 8 days-old mice.

Assay of the pentobarbital sleeping time in CF-I mice treated with SKF525A suspended in blood. Sodium pentobarbital (40 mg/kg in 0.9% NaCl) was injected i.p. When the animals (5 male

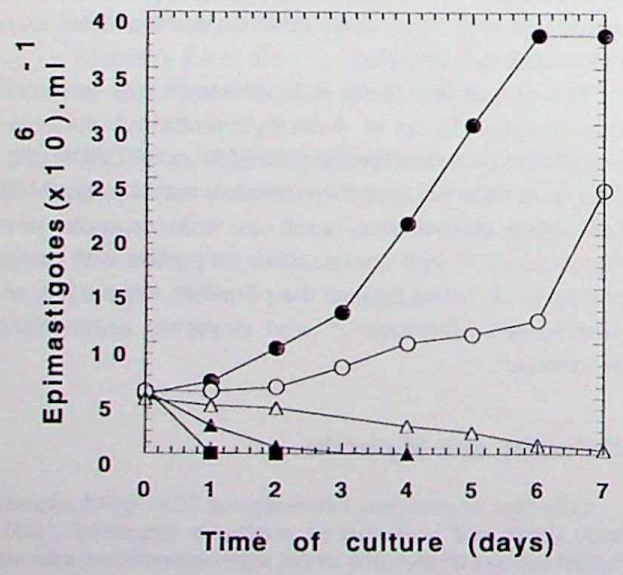

Fig. 1.– Effect of SKF525A on growth of epimastigotes, Tul2 stock, in axenic culture. The drug concentrations used were 0 (●), 25 (○), 50 (Δ), 75 (▲) and 100 (■) µM.

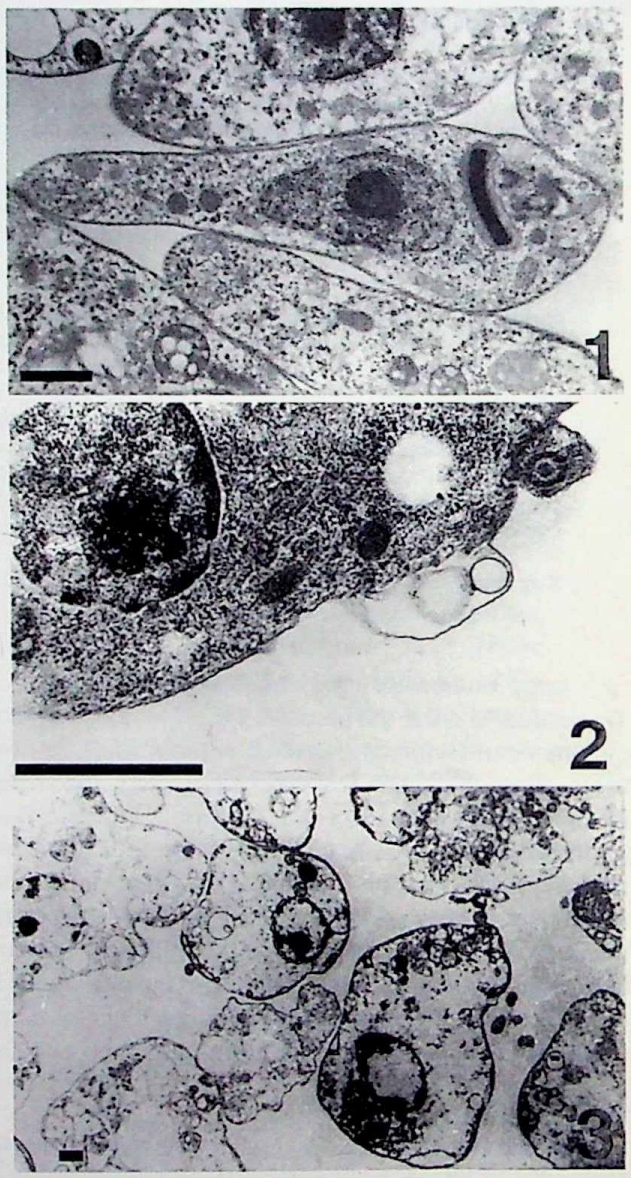

Fig. 2.– Effect of SKF525A on the ultrastructure of *Trypanosoma cruzi* epimastigotes. The epimastigotes were incubated at 28°C in the absence (1) or in the presence of 0.1 mM SKF525A for 164 min, (2) or 360 min, (3) Samples were processed for electron microscopy as described under Materials and Methods 1, x 14 000; 2, x 36 500; 3, x 3 600.

mice per group) got asleep, blood treated with or without 0.5 mM SKF525A for 24 hr at 4°C (0.1 ml/20 g body weight) was given i.v., and the sleeping time was recorded.

Electron microscopy. Epimastigotes were fixed by suspension in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min. at room temperature. Afterwards samples were treated as described[14], and thin section were observed in a Philips EM 300 electron microscope.

Chemicals. SKF525A was obtained from Smith, Kline and French Labs, Philadelphia, PA, U.S.A. All other chemicals used were analytical reagents of the highest purity available.

Results and discussion

Incubation of epimastigotes (Tul 2 or RA strains) or cell-culture trypomastigotes (RA strain) with 0.1 mM SKF525A led to their complete immobilization in 360, 160 or 18 min., respectively. The presence of the drug during axenic culture of Tul 2 epimastigotes led to complete inhibition of growth and progressive parasite lysis even at a concentration as low as 50 mM SKF525A (Fig. 1). Incubation of the epimastigotes with the drug resulted in progressive damage to cellular structure (Fig. 2). After 6 hr the cells looked almost like empty bags, with damage to the plasma membrane. The kinetoplast and the subpellicular microtubules were relatively little affected. This suggests that, at variance with other amphiphilic drugs, like trifluoperazine[14], the mitochondrion is not the primary target of SKF525A.

When the drug was tested on the parasite cycle in cultured Vero cells (Table 1) the presence of SKF525A during infection and subsequent culture caused a marked

TABLE 1.– ***Effect of SKF525A on the parasite cycle in Vero cells.*** *The experiment was performed as described under Materials and Methods. a) Drug present during infection and subsequent culture; b) Drug present only during infection; c) Drug added 24 hr after infection; d) Trypomastigotes pre-incubated for 4 hr with 10 µM SKF525A before infection, which was performed at the drug concentrations stated in the Table.*

Treatment	Infected cells %	Amastigotes/cell	Endocytic index
Control	11.9 ± 1.3	16.4 ± 14.3	196
5 µM SKF525A			
a	5.6 ± 1.1	8.8 ± 10.4	49
b	6.3 ± 0.02	10.1 ± 9.2	64
c	13.8 ± 1.9	11.1 ± 10.6	153
d	6.3 ± 0.09	8.8 ± 9.9	55
10 µM SKF525A			
a	2.6 ± 0.2	5.3 ± 4.9	14
b	5.1 ± 0.1	10.7 ± 9.0	54
c	13.6 ± 1.6	10.8 ± 9.6	147
d	1.1 ± 0.36	5.1 ± 3.6	6

TABLE 2.– *Effect of SKF525A on Tul 2 strain bloodstream trypomastigotes in mouse blood. The experiment was performed as described under Materials and Methods. The results are given as average of 5 determinations ± SE*

Concentration of SKF525A (mM)	Parasitemia (tryps/ml x 10^4) at day p.i.						Mortality (at day p.i.)
	6	10	13	17	20	25	
0	7.67 ± 0.17	258 ± 10.5	-	-	-	-	100% (12)
0.3	0	26.3 ± 9.9	147.5 ± 57.9	-	-	-	100% (16)
0.4	0	6 ± 5.9	76.2 ± 45.5	215	-	-	100% (18)
0.5	0	3.2 ± 1.8	23 ± 10	198 ± 69.6	-	-	100% (19)
0.6	0	0	0	0	0	0	0%
0.8	0	0	0	0	0	0	0%
1.0	0	0	0	0	0	0	0%

decrease in the endocytic index in a concentration dependent manner. Considerable decrease was also observed when the drug was present only during infection, or when the trypomastigotes were preincubated with 10 µM SKF525A. On the other hand, when the drug was added 24 hr after infection, while changing the culture medium, there was little effect on the endocytic index. These results suggest that the effect of SKF525A is primarily on the infecting trypomastigotes, and that the drug is not able to affect the intracellular parasite growth.

Aiming at a possible application of SKF525A or related drugs for the sterilization of banked blood samples, two different experiments were performed. First, cell-culture trypomastigotes (RA strain) were suspended in mouse blood, incubated for 24 hr at 4°C with variable concentrations of drug, the surviving parasites were counted in a Neubauer chamber, and the blood was used to infect Vero cell cultures, as described under Methods. The control without drug contained 1.68 x 10^5 parasites/ml, and the Vero cells inoculated with this sample released trypomastigotes into the medium after 6 days in culture. Parasite numbers were reduced to 0.5 x 10.5^5 parasites/ml in the presence of 0.1 mM SKF525A, and to 0 at concentrations of 0.2 mM or higher. At 0.2 mM, very few trypomastigotes were released after 12 days; at 0.3 mM only after 20 days, and at drug concentrations of 0.4 mM or higher no parasites were observed in the cell cultures.

Table 2 shows the results of a typical experiment in which blood from mice infected with the Tul 2 strain was incubated for 24 hr at 4°C with increasing concentrations of the drug, and then inoculated into 8-days old BALB/c mice, five per drug concentration. Control mice showed the parasitemia peak at 10 days p.i., and all died at day 12 p.i. Concentrations of SKF525A from 0.3 to 0.5 mM increased in a dose-dependent manner the time required for the parasitemia peak and 100% mortality, and finally 0.6-1.0 mM SKF525A completely prevented the development of patent parasitemia, all the animals surviving for up to 30 days. The survivors were sacrificed, and homogenates of heart or liver, and blood, were inoculated into 8 days-old mice, which showed no parasitemia up to 60 days. SKF525A concentrations up to 1 mM did not damage the red blood cells (the cells looked indeed better than the controls), and caused no apparent damage to mice injected with non-infected blood containing the same drug concentrations. Since the drug is a well-known inhibitor of mammalian cytochrome P450[15], it was necessary to test the possibility that SKF525A concentrations carried over with the injected blood might have deleterious effects, for instance increasing the effects of anesthetics. However, the pentobarbital sleeping time recorded for the control animals was 36 ± 19 min., and that for the animals inoculated with 0.1 ml/20 g of body weight of 0.5 mM SKF525A in mouse blood was 35 ± 28 min., without significant difference (P = 0.91).

The results presented herein suggest that some inhibitors of the cytochrome P450 might be tested as potentially useful additives for their use in blood banks.

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