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INHIBITORY ACTION OF A 5-NITROFURAN (SQ18506) AGAINST NUCLEIC ACID SYNTHESIS IN *TRYPANOSOMA CRUZI*

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Abstract—The mode of action of SQ18506 (a 5-nitrofuran) against *Trypanosoma cruzi*, a protozoan, was found in previous studies to be inhibition of nucleic acid synthesis. To find out how this inhibition occurred, we systematically investigated the effect of the drug on three components of nucleic acid synthesis: uptake and metabolism of nucleic acid precursors, nucleic acid polymerases, DNA template. *In vitro*, SQ18506 inhibited both DNA and RNA polymerases and interacted with DNA. Thus, at physiological drug concentrations there was marked inhibition of the polymerases which was increased in the presence of a nitro-reducing system. SQ18506 also decreased the T_m of calf thymus DNA by up to 5°. *In vivo*, the drug caused single strand breaks within 1 hr of incubation and double strand breaks by 7.5 hr in the DNA of *T. cruzi*. Thus, inhibition of nucleic acid synthesis may be the result of a combination of lesions, though whether it is actually responsible for the cell lysis which is the culmination of the effect of the drug remains undecided.

Trypanosoma cruzi is the pathogenic protozoan that causes Chagas' disease, the incurable South American trypanosomiasis. The disease in experimental animals is sensitive to 5-nitrofurans (5-NF), the most active of which is SQ18506 (trans-5-amino-3-[2-(5-nitro-2-furyl)vinyl]-Δ^2-1,2,4-oxadiazole). Previously, it was found that SQ18506 acted against *T. cruzi* by inhibiting nucleic acid synthesis [1]. We have now investigated this inhibition in depth to find biochemical differences between host and parasite, so that drugs might be designed more rationally.

Other 5-NF and 5-nitroimidazoles (5-NI) that are active against trichomonads and bacteria also inhibit nucleic acid synthesis. Generally, the synthesis of DNA, RNA and protein is inhibited very rapidly at low drug concentrations, with DNA synthesis being the most sensitive. Most work concerning the molecular action of 5-NF has been performed by McCalla and coworkers [2, 3, 4, 5, 6]. They have shown that several 5-NF cause single strand breaks (SSB) in bacterial and mammalian DNA. The production of these was correlated to the reduction of the nitro group and also to the mutagenicity of the drug. At least two types of lesions could be detected in the super coiled DNA of *Escherichia coli* minicells [5]. One type was detected in neutral sucrose gradient centrifugation and the other by alkaline gradient centrifugation. Like most SSB, the breaks detectable in neutral sucrose were rapidly repaired, but the alkaline detectable breaks were not [5].

It is difficult to reconcile the selective action of 5-NF with their lack of selectivity in causing damage to both bacterial and mammalian DNA. Indeed, SSB production is possibly the basis of the mutagenic and carcinogenic action of 5-NF. What has yet to be explained is how nitrofurantoin, a noncarcinogen and low-intensity mutagen, is actively anti-bacterial [3]. The reason may be that 5-NF have actions other than on DNA that may rapidly inhibit macromolecular synthesis. Such actions include the binding to and interference with the function of bacterial ribosomes [7, 8, 9]. It has also been reported that a 5-NF can inhibit DNA polymerase in *E. coli* [10] and a 2-NI ribonucleotide reductase in *Bacillus subtilis* [11].

Possibly the selectivity may reside in differential drug metabolism. Evidence has been produced that the active drug species of 5-NF action is a reduced metabolite, either the hydroxylamino form [12] or a radical anion derivative [13].

To investigate how SQ18506 inhibits nucleic acid synthesis in *T. cruzi*, we examined the effects of the drug on the three key parameters: precursor uptake and metabolism, DNA template functioning, and the nucleic acid polymerases.

MATERIALS AND METHODS

Materials

Chemicals were obtained from Fisons Ltd, Leicester: biochemicals from Sigma Ltd., Kingston-Upon-Thames, and radiochemicals from the Radiochemical Centre, Amersham. Epimastigote forms of *T. cruzi* were grown axenically in undefined LIT medium with 10 per cent new-born calf serum (Flow Laboratories, Irvine, Scotland) at 28° [14]. The cultures were used when in the logarithmic phase of growth.

Methods

Precursor uptake and metabolism. For uptake studies, trypanosomes (about 5×10^7/ml) were incubated with the appropriate precursor [8-^{3}H]adenine (1 μCi/ml, specific activity 25 Ci/mmoles) or [4,5-^{3}H]-leucine (1 μCi/ml, specific activity 54 Ci/mmoles) in Krebs–Ringer glucose solution. At intervals, 10 ml

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Non-standard abbreviations NF–Nitrofuran; NI–Nitroimidazole; SSB–Single strand break; T_m–Melting temperature; SDS–Sodium dodecyl sulfate.

samples were removed and thrice washed. Then the cellular macromolecules were precipitated with cold 5% (w/v)-CCl_3CO_2H, the supernatant fraction removed by centrifugation, and estimated for radioactivity by scintillation counting using a water-miscible scintillation fluid. Thymidylate synthase was assayed by the method of Lomax and Greenberg [15] as modified by Al Chalabi and Gutteridge [16].

Effect on DNA. The effect of SQ18506 on the thermal denaturation of calf thymus DNA was investigated *in vitro*, the procedure of Mandel and Marmur [17] being followed. The DNA (20 μg/ml) was heated in 0.1 × SSC (standard saline citrate) at 0.5°/min (25–50°) and then 0.25°/min (50–90°). The results are corrected for the increase in absorbance of the SQ18506 (1–2 μg/ml) solution.

In vivo experiments were conducted by incubating the trypanosomes in SQ18506-containing medium and then studying their DNA. Electron microscopic examination of the kinetoplast DNA (kDNA) and sedimentation of nuclear (nDNA) and kDNA in CsCl gradients used conventional techniques (18, 19). For studies of the sucrose gradient centrifugation of *T. cruzi* DNA, organisms were incubated in [6-^{3}H]-thymidine for 18 hr and then washed. For alkaline sucrose gradients, trypanosomes (about 1 × 10^6) in 25 μl of isotonic saline were pipetted into 250 μl of lysing solution (500 mM-NaOH, 1% (w/v)-SDS, 300 mM-EDTA and 100 mM-NaCl, pH > 12) which had previously been layered onto a linear sucrose gradient (5–20% (w/v)) of 4.5 ml. The gradient contained sucrose, 100 mM-NaOH, 300 mM-EDTA and 100 mM-NaCl. After 20–60 min (100% cell lysis) the gradient was centrifuged for 1–2 hr at 30,000 rpm in the MSE SS65 centrifuge using a 3 × 6.5 ml swing-out rotor. After centrifugation, fractions of 200–250 μl were collected from the bottom of the gradient and estimated for radioactivity by scintillation counting using a toluene/2-methoxyethanol based fluid.

For neutral gradients 1 × 10^6 trypanosomes in 25 μl of Krebs–Ringer glucose solution were lysed for 10–60 min in 250 μl of a mixture of 1% (w/v)-SDS, 1% (w/v)-Na deoxycholate, 100 mM-EDTA, 150 mM-NaCl and 100 mM-Tris–HCl buffer, pH 7.5. This had been layered on a gradient of 4.5 ml of 5–20% (w/v) sucrose, 100 mM-EDTA, 150 mM-NaCl, 0.5% (w/v)-SDS and 100 mM-Tris–HCl buffer pH 7.5, which was centrifuged for 18 hr at 8000 rpm in the 3 × 6.5 ml rotor and processed as for alkaline gradients.

Nucleic acid polymerases. The effect of SQ18506 on the polymerases was estimated using partially purified enzymes. The RNA polymerases were isolated using techniques described for trypanosomes (20, 21, 22). This procedure used disruption by homogenisation and sonication, ammonium sulfate fractionation and DEAE cellulose chromatography. Using a linearly increasing ammonium sulfate gradient (0.03 to 0.8 M), three peaks of RNA polymerase activity were eluted from the DEAE column. The assay mixture (100–120 μl) consisted of 50–80 μl of enzyme extract, 10 μg of calf thymus DNA, 500 μg of bovine serum albumin, 500 mM-tris HCl buffer pH 7.9, 300 μM-GTP, -CTP, -ATP, 200 μM-[5-^{3}H]-UTP (specific activity 100 Ci/mol), 2 mM-dithiothreitol, 10 mM-$MgCl_2$, 10 mM-$MnCl_2$, 15% (v/v) glycerol and 20 mM-NaF. After incubation at 28° for 60 min the macromolecules were precipitated with 2 ml of cold 5% (w/v)-CCl_3CO_2H. After standing at 4° for 30 min the precipitate was filtered onto a glass fiber filter, washed with 20 ml of cold 5% (w/v)-CCl_3CO_2H, 5 ml of cold 70% (w/v) ethanol and the radioactivity estimated.

The DNA polymerase was assayed in the 100,000 *g* supernatant fractions of *T. cruzi* homogenates. The assay mixture (100–120 μl) consisted of 20–80 μl of enzyme extract, 50 mM-Tris–HCl buffer-pH 7.5, 50 μM-dATP, -dGTP, -dCTP, 25 μM-[methyl-^{3}H]-dTTP (specific activity 80 Ci/mole), 5 mM-$MgCl_2$, 10 μg activated (by the method of Aposhian and Kornberg [23]) calf thymus DNA, 10 μg denatured (70° for 5 min followed by rapid cooling in ice) salmon sperm DNA, 2 mM-EDTA, 20 mM-NaF, 20 mM-mercaptoethanol and 500 μg of bovine serum albumin. The assay mixture was incubated at 28° for 20 min and processed as for RNA polymerase.

RESULTS

Purine and pyrimidine uptake. Culture epimastigote forms of *T. cruzi* are unable to synthesise purines *de novo* and are therefore dependent on the salvage of exogenous purines for nucleic acid synthesis [24]. Under the experimental conditions used in the precursor uptake experiments, little nucleic acid synthesis occurred in control incubations (< 1 per cent of normal), thus dissociating purine uptake and nucleic acid synthesis. This was necessary so that inhibition by SQ18506 of the incorporation of these precursors into nucleic acids did not lead to accumulation of the precursors in the cell and consequential inhibition of uptake unrelated to any effect of SQ18506 on the actual uptake process. Even so, the uptake of [^{3}H]-adenine into the cold CCl_3CO_2H soluble fraction was rapid and linear, and SQ18506 had no effect on the uptake in the 80 min of incubation (Fig. 1). This contrasted with prior studies which had shown that nucleic acid synthesis itself was inhibited within this period [1]. However, this observation is in agreement with previous studies that showed that the drug did not affect intracellular ATP concentrations [1].

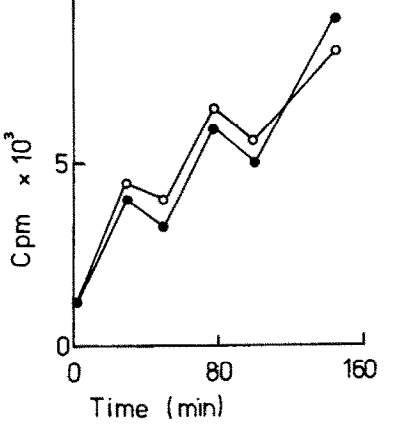

Fig. 1. Effect of SQ18506 on the uptake of [^{3}H]-adenine into the cold 5% (w/v)-CCl_3CO_2H soluble fraction of *T. cruzi* epimastigotes. Drug was added at 10 min. (●) control, (○) SQ18506 30 μg/ml.

Table 1. Effect of SQ18506 on thymidylate synthase

	SQ18506 (μg/ml)	Per cent of control
In vivo		
T. cruzi (3×10^8/ml) maintained in SQ18506 containing medium for 4 hr	0	100
	20	95 (1)
	100	100 (1)
	500	71 (1)
In vitro	0	100
	100	96 (1)
	500	106 (1)

In vivo the organisms were incubated, then disrupted, and the activity in the homogenate estimated. *In vitro*, the activity was estimated in normal trypanosomal homogenates.

T. cruzi is able to obtain pyrimidines both by synthesis *de novo* and by salvage [24]. SQ18506 was thought unlikely to affect this area, since to be effective it would need to block both pathways simultaneously. *In vivo*, deoxythymidylate is obtained predominantly by *de novo* synthesis [25]. However, SQ18506 did not substantially inhibit the thymidylate synthase of *T. cruzi* (Table 1).

Thus it was concluded from this section of the investigation that it was unlikely that SQ18506 blocked nucleic acid synthesis by interfering with the salvage and/or syntheses of purines and pyrimidines.

Action on DNA. SQ18506 (1 μg/ml) decreased the T_m (melting temperature) of the calf thymus DNA by up to 5° and increased the total absorbance (Fig. 2). This effect was observed even after correcting for absorbance changes of SQ18506 solutions, but occurred only at pH 8.5 and not pH 7.0. One interpretation is that the drug decreased inter-strand binding.

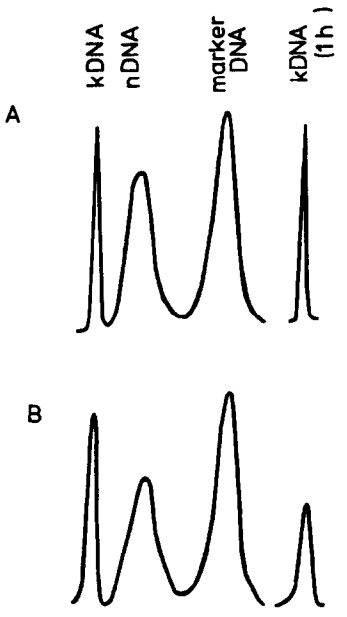

Fig. 3. Centrifugational properties of DNA from *T. cruzi* incubated in SQ18506-containing medium. A is DNA from trypanosomes after 2 hr and 6 hr. B was DNA from trypanosomes incubated in SQ18506 (100 μg/ml) containing medium for 6 hr. The marker DNA is *Micrococcus lysodetikus* of density 1.731 g/ml. The density of the kDNA was 1.6976 and nDNA 1.7087. The kDNA rapid banding was assessed after 1 hr.

Isopycnic CsCl ultracentrifugation of detergent lysates of *T. cruzi* have indicated the presence of two DNA components—a nuclear component of density 1.709 g/ml and a kinetoplast component of 1.698 g/ml. This latter component, which is thought to be the equivalent of mitochondrial DNA, is released from detergent lysates of trypanosomes as a single net-

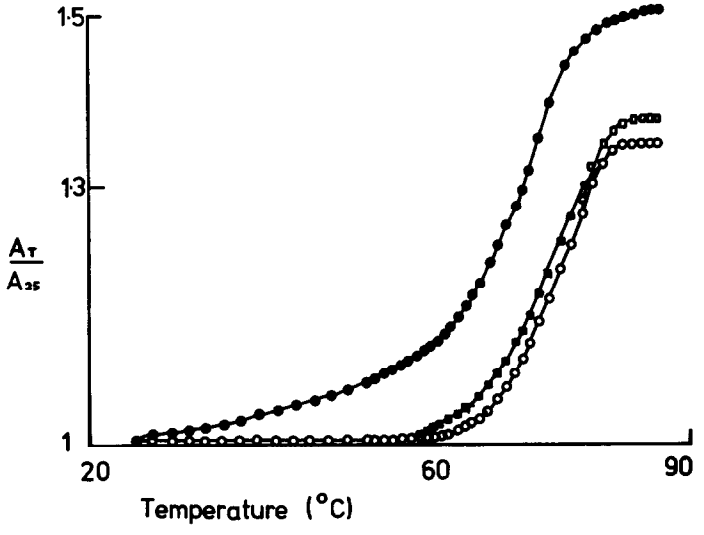

Fig. 2. Effect of SQ18506 on the thermal denaturation of calf thymus DNA, (○) DNA-pH 7.0 and DNA/SQ18506-pH 7.0, (■) DNA-pH 8.5, (●) DNA/SQ18506-pH 8.5, (□) DNA/SQ18506-pH 7.8. The results are plotted as

$$\frac{\text{Absorbance at temperature T}}{\text{Absorbance at 25°}}$$

against temperature *T*.

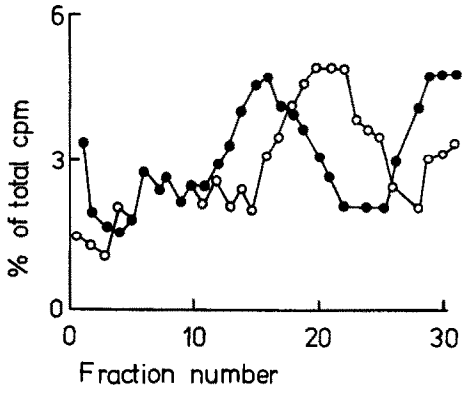

Fig. 4. Alkaline sucrose gradient centrifugation of DNA from *T. cruzi* epimastigotes incubated in SQ18506-containing medium for 1 hr. Fraction 30 is the top of the gradient. (●) DNA from control trypanosomes, (○) DNA from test trypanosomes.

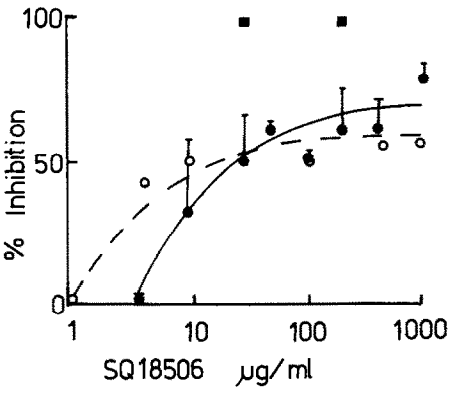

Fig. 6. Inhibition of RNA polymerase 1 (●) and 3 (○) by varying concentrations of SQ18506. Bars represent standard error of the mean. Closed squares represent the inhibition by SQ18506 in the presence of a nitro-reducing system.

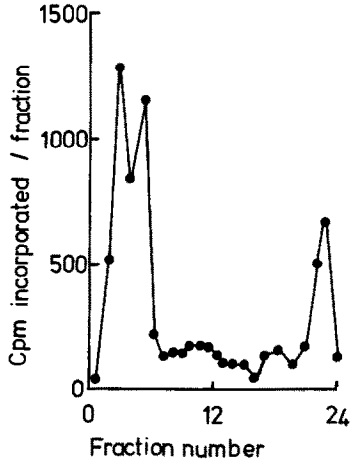

Fig. 5. Typical elution pattern of RNA polymerases from DEAE cellulose chromatography. The peaks were named 1, 2 and 3 according to the order of their elution.

Table 3. Effect of omitting components and different templates on the activity of DNA polymerase of *T. cruzi*

Specific activity 36.1 ± 14.1 (8) pmol dTMP incorporated/min/mg protein	
	Per cent of control
Minus enzyme	20 (2)
Minus DNA	10 (2)
Minus dCTP	20 (1)
Plus DNase	24 (2)
Plus ethidium bromide (100 μg/ml)	30 (2)
Denatured salmon sperm DNA	145 (2)
Nicked DNA (45 min incubation)	80 (1)
Nicked DNA (120 min incubation)	62 (1)
Sonicated DNA	132 (1)

Results expressed as in Table 2.

Table 2. Characterisation of RNA polymerase of *T. cruzi*

	Position off column		
	1	2	3
Initial specific activity*	22 ± 1 (5)	0.181 (2)	15.6 ± 8 (3)
Final specific activity*	160 (2)		550 (1)
	Per cent of control		
Minus enzyme	31.6 ± 29 (4)		
10 μl enzyme†	28.8 ± 25 (5)		
20 μl enzyme†	26 ± 15 (3)		
50 μl enzyme†	39 (2)		
Minus DNA	16.1 ± 7 (7)	0 (1)	30 (2)
Minus GTP	19.5 ± 9 (4)	0 (1)	23 (2)
Plus RNase (1 mg/ml × 30 min)	0 (2)	0 (1)	0 (2)
Plus ethidium bromide (100 μg/ml)	0 (2)		
Denatured DNA as template	50 (1)		

* pmol UMP incorporated/min/mg protein.

† 100 per cent activity is with 80 μl of enzyme in assay system.

Experiments were all performed in duplicate and the results represent the mean of these duplicate determinations ± the standard deviation, with the number of experiments in brackets.

work. It thus bands rapidly in CsCl gradient and the band that is formed is very sharp because the networks are very homogeneous in size [25]. The DNA patterns of *T. cruzi* from cultures incubated for 2 hr or 6 hr with physiological concentrations of SQ18506 were unaltered. However, after 6 hr of incubation at a high drug concentration, it was observed that both the nDNA and kDNA peaks broadened and that the rapid banding of the kDNA peak was reduced (compare Fig. 3A and B). No changes were visible in the appearance of this kDNA in the electron microscope.

Using alkaline sucrose gradients, it was found that SQ18506 (at physiological concentrations) caused SSB in the DNA of *T. cruzi* after 1 hr incubation. This was shown when the DNA (labelled with [^{3}H]-thymidine) of test organisms did not travel as far down the sucrose gradient as labelled DNA of control organisms (Fig. 4). Additionally, it caused double strand breaks, but only after 7.5 hr incubation. Again, this was shown by the fact that labelled DNA of test organisms did not pass as far down a neutral sucrose gradient as did that of control DNA.

Nucleic acid polymerases. The RNA polymerase activity could not be assayed in crude homogenates, presumably due to the presence of RNase contamination. The procedures used to purify the enzymes were the minimum necessary to demonstrate RNA polymerase activity. From the DEAE column three peaks of activity were eluted (Fig. 5). Even when isolated the polymerases were unstable and 15% glycerol (v/v) was the best stabilising agent for RNA polymerase 1. The isolation and assay conditions were continuously improved during the period of experimentation, as reflected in the increase in specific activity, and the characteristics of the enzymes are given in Table 2. The effect of the drug on the RNA polymerases is shown in Fig. 6. SQ18506 (at physiological concentrations) inhibited all three polymerase enzymes. The addition of a nitro-reducing system (5 units xanthine oxidase and 10 μmol hypoxanthine) increased the inhibitory action of the drug.

The effect of SQ18506 was estimated against the DNA polymerase in the 100,000 g supernatant of *T. cruzi* homogenates. The characteristics of the enzyme are given in Table 3 and the effect of SQ18506 in Fig. 7. The drug caused inhibition of the DNA polymerase, and in the presence of a nitro-reducing system this was increased.

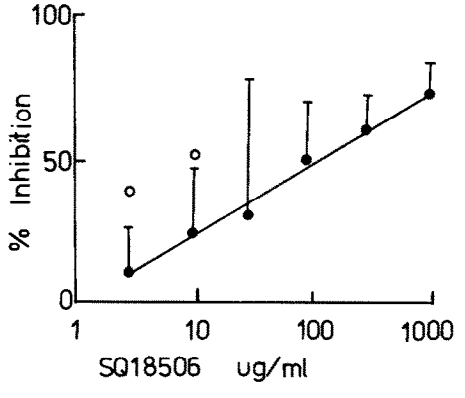

Fig. 7. Inhibition of DNA polymerase by SQ18506. Bars represent standard error of mean. Open circles represent the inhibition by SQ18506 in the presence of a nitro-reducing system.

DISCUSSION

Like previous workers, we found that a 5-NF (SQ18506) causes damage to DNA *in vivo*; in this case the damage was towards both nDNA and kDNA. It was manifested by alterations in the centrifugational patterns of the DNA. In addition, it was shown that the antimicrobial selectivity of SQ18506 and other 5-NF might be due to the inhibitory activity of the drug on the nucleic acid polymerases. Previously, this possibility has only been briefly examined, as it was assumed that the inhibition of nucleic acid synthesis was caused by 5-NF-induced damage to DNA.

Though it was not proved conclusively that SQ18506 acted on the polymerases *in vivo*, in an initial experiment there was no detectable DNA polymerase in 100,000 g supernatant fraction of *T. cruzi* maintained in medium containing SQ18506 for 4 hr. It is interesting that many alkylating agents have now been found to have a dual action—on the DNA and nucleic acid polymerases [26, 27].

However, 5-NF do have a selective action, for some 5-NF have been found to selectively increase the extractable RNA polymerase of rat liver [28].

Thus the effects of SQ18506 and other 5-NF are similar to other alkylating agents including another nitro compound, 4-nitroquinoline-N-oxide. This compound is a mutagen and carcinogen, and causes repairable SSB in DNA [29, 30]. It is also active against *T. cruzi* and Leishmania (another protozoan) [31, 32]. Against *T. cruzi* the kinetics of the compound's action were similar to that of SQ18506, and it is also likely that a reduced species is the active form [33].

In this context it was interesting that a nitro-reducing system increased the inhibitory action of the SQ18506, though it should be noted that the SQ18506 inhibited the polymerases in the absence of a drug reducing system, possibly by the same mechanism responsible for the lowering of the T_m of calf thymus DNA .*T. cruzi* has been shown to reduce the nitro-group of SQ18506 [1].

Thus the precise mode of inhibition by SQ18506 of nucleic acid synthesis is uncertain. What is clear is that the drug may act on the DNA template or nucleic acid polymerases or a combination of both. This is important, for there are no prior studies on the biochemical action of 5-nitrofurans on protozoans.

Whether the inhibitory action of SQ18506 against nucleic acid synthesis causes cellular lysis is uncertain; for SSB are generally repairable, and it is difficult to comprehend how inhibition of protein synthesis can cause cell lysis. Additionally, ethidium bromide (a DNA intercalating drug) takes longer than 24 hr to cause lysis of *T. cruzi* [34] (cf. 6 hr in the case of SQ18506). However, SQ18506 and other 5-NF do have a multiplicity of actions, one of which may be responsible for cell lysis.

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