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# NUCLEOTIDE AND POLYNUCLEOTIDE SYNTHESIS IN *TRYPANOSOMA CRUZI*

## V — Effects of primaquine, stylomycin derivatives and analogs, on experimentally infected mice

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**SUMMARY**

The treatment of mice infected experimentally with *T. cruzi* by the aminonucleoside of stylomycin and primaquine was found to be very effective. The association of the drugs bringing about:

1. Total survivals of the infected animals when 100% of death occurred among the untreated animals.
2. Remarkable lowering or absence of blood parasitism.
3. Pronounced favourable histopathological effects on the infection of the heart and skeletal muscles and no toxic effects on the tubular cells of the kidney.

The effect of each drug administered separately was found to be less pronounced than the association, and in addition necrotic changes of the renal tubular cells were observed after the prolonged administration of the aminonucleoside of stylomycin.

Very little (if any) chemotherapeutic effect was found when the animals were treated with the $N_6$ diethyl analogue of stylomycin or the $N_6$ dimethyladenine.

## INTRODUCTION

The discovery by HEWITT *et al.*[6] that the antibiotic stylomycin * exerts a pronounced trypanostatic activity against *Trypanosoma equiperdum* and a variable activity against *Trypanosoma cruzi* has aroused considerable interest. They showed that the antibiotic was very effective against experimental mouse infection by *T. equiperdum*, and TOBIE [9] found a similar effectiveness of the drug against six species of African trypanosomes in mice. HEWITT *et al.*[5] showed furthermore that the chemotherapeutic effects of stylomycin and its aminonucleoside derivative on *T. equiperdum* could be reversed by purines, suggesting that the drug might interfere in some way with the meta-

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* Stylomycin is the trade name of Lederle Laboratories Division, American Cyanamid Co., for the antibiotic formerly known as Achromycin. This name was changed subsequently to Puromycin and afterwards to Stylomycin.

bolism of purines of the parasite. These findings stimulated further studies on how *T. cruzi* makes its purine compounds, in order to get more fundamental knowledge and incidentally to apply it as a rational basis for a chemotherapeutic approach.

Following this line, FERNANDES & CASTELLANI [2] have shown that the usual forms of *T. cruzi* as obtained in cultures on the common diphasic media, cannot make the purine ring, using therefore preformed bases to build up their purine nucleotides and polynucleotides. They showed furthermore that the antibiotic stylomycin did not inhibit this utilization of preformed purines. On the other hand, a fragment of the antibiotic, its aminonucleoside, was found to inhibit the synthesis of the purine compounds (FERNANDES & CASTELLANI [1]) by the same form of parasites. Furthermore, on the intracellular forms found in the cycle of the parasite in tissue cultures, this compound acts promoting an intense and remarkable degeneration of the reproductive forms of this protozoon (SILVA, YONEDA & FERNANDES [8]). The effect of this drug in experimental mice infection was found to be very pronounced FERNANDES, PEREIRA & SILVA [3]) as detected by the lowering of the blood parasitism and by the prevention of death in the treated animals. The surviving animals, however, were found to harbor the parasite.

Since the most remarkable effect of the aminonucleoside of stylomycin appeared to be on the intracellular leishmania in tissue culture, and primaquine (PIZZI [7]) seems to act on the extracellular form, an assay associating both drugs was undertaken. The present paper is concerned with the results obtained assaying this association, and observations on the infected animals tolerance for the aminonucleoside of stylomycin as well as the effects of one analogue and one derivative of this aminonucleoside, namely $N_6$ diethylaminonucleoside and $N_6$ dimethyladenine respectively, on the experimental mice infection by *T. cruzi*.

## MATERIAL AND METHODS

Young mice weighing around 15 g (12-17 g) were inoculated intraperitoneally with a virulent strain of *T. cruzi* (500,000 organisms to each mouse). The treatment started 3 hours after the inoculation and prolonged up to 68 days except in the period between the 21st and 38th day of infection. Each mouse received 4 daily injections (subcutaneously) of the drug assayed or saline throughout the treatment schedule. The daily doses used per Kg of body weight were: aminonucleoside (26 mg), $N_6$ diethylaminonucleoside (17 mg), $N_6$ dimethyladenine (15 mg) and primaquine (13 mg). We must add that on a molecular basis an equal dose of the aminonucleoside of stylomycin, its $N_6$ diethyl analogue and $N_6$ dimethyladenine were employed. Unless when indicated the treatment schedule and technique for blood parasite counting and xenodiagnosis was the one reported by FERNANDES, PEREIRA & SILVA [3]. Four criteria were used to evaluate the effectiveness of the treatment: blood level parasitism; the amount of intracellular parasites in heart and skeletal muscles; the histopathological reactions in these organs and kidneys and the survival time of the infected animals. For the histopathological studies 30 animals were used. All 5 animals of the untreated group were examined soon after their death, which occurred 13 to 15 days after the infection by *T. cruzi*. From nine mice treated with dimethyladenine, 5 were examined after deaths that occurred 13, 16, 20, 20 and 26 days after the injection and the remaining 4 were sacrificed 14, 14, 14 and 74 days after the *T. cruzi* inoculation. Nine animals treated with the aminonucleoside of stylomycin were examined. In order to follow the drug toxicity to the kidneys, 3 animals were sacrificed on the 14th day of infection and the remaining 6 were examined after sacrifice on the 74th day after the *T. cruzi* inoculation. All 3 animals treated with primaquine were sacrificed on the 14th day of infection. Four animals were examined among those treated with primaquine plus the aminonucleoside of stylomycin. Three of them were sacrificed on the 14th day and 1 on the 31st day of infection. The technique used was Bouin fluid fixation after which fragments of heart, skeletal muscle of 2 or 3 different regions and half of each kidney were embedded in paraffin. From the heart 2 transverse fragments of the whole organ

were taken, one at the level of the atrium and the other at the superior part of the ventricles.

## RESULTS

The effects of the drugs on the survival time as well as on the number of blood parasites are indicated in Table I. It can be observed that the diethyl analogue of the aminonucleoside and $N_6$ dimethyladenine affect very little the evolution of the infection as judged by the survival time of the mice infected. However, $N_6$ dimethyladenine seems to lower the blood parasitism. On the other hand, the aminonucleoside of stylomycin and primaquine were found to be very effective since no deaths occurred among the animals treated with either drug. It can be seen that during the treatment period primaquine seems to be more effective than the aminonucleoside of stylomycin, in relation to the cleaning up of the blood parasite. Once the drug administration is withdrawn, the level of blood parasites rises in those animals previously treated with primaquine, thus confirming the results showing that the drug affects mainly the extracellular parasites (PIZZI [7]). On the other hand, those animals treated with the aminonucleoside of stylomycin showed very few parasites in their blood, even after the withdrawal of treatment. However, the animals continue to harbor blood parasites as found through xenodiagnosis, confirming in this way the results of FERNANDES, PEREIRA & SILVA [3]. The association of the aminonucleoside of stylomycin seemed to be highly effective because the blood parasitism must be very low, since in only one out of 5 mice was it found to be positive through xenodiagnosis.

TABLE I

Chemotherapy of experimental infection in mice by *Trypanosoma cruzi*. Each group consists of ten mice and each animal received four daily injections of the indicated drugs, from three hours after inoculation up to 68 days after, except for the period between the 21st. and 38th. day.

| *Drugs* (mg/kg body weight/day) | *Survival* | | *Number of parasites per mm³ of blood on the indicated days after the inoculation* | | | | *Xenodiagnosis (on the 70th. day of infection)* |
|---|---|---|---|---|---|---|---|
| | *% of survivors* | *Average survival time in days* | 9th. | 14th. | 32th. | 57th. | |
| Controls (saline) | — | 12.5 | 8,400 | 17,000 (1 animal) | (*) | (*) | ... |
| $N_6$ diethyl analogue (17 mg) | 20 | 14.0 | 4,700 | 1,220 | Positive 100% | Positive 100% | ... |
| $N_6$ dimethyladenine (15 mg) | 20 | 18.6 | 435 | 650 | Positive 100% | Positive 100% | ... |
| Aminonucleoside of stylomycin (26 mg) | 100 | (**) | 180 | 53 | Positive 100% | Positive 50% | ... |
| Primaquine (13.3 mg) | 100 | (**) | 20 | 15 | 275 | Positive 90% | ... |
| Aminonucleoside + Primaquine | 100 | (**) | — | — | — | Negative 100% | Negative<br>Negative<br>Negative<br>Positive |

(*) No mice alive at is time.
(**) All survivors appeared healthy when they were killed after four months of infection with *T. cruzi*.

TABLE II

Intracellular parasites and histopathological findings in mice infected by *Trypanosoma cruzi* and treated with primaquine, aminonucleoside of stylomycin and $N_6$ dimethyladenine. The extension of the histopathological findings as well as the intensity of the intracellular parasitism is directly proportional to the number of plus (+).

| *Treatment* | *No. of mice* | *Heart* | | *Skeletal muscle* | | *Kidney tubular necrosis cortical atrophy* |
|---|---|---|---|---|---|---|
| | | *Amount of intracellular parasites* | *Inflammatory reaction* | *Amount of intracellular parasites* | *Inflammatory reaction* | |
| Control | 5 | ++++ | +++ | ++ | + | — |
| Dimethyladenine | 9 | ++++ | ++++ | ++ | + | — |
| Amino on the 14th. day of treatment | 3 | +++ | ++ | — | + | — |
| Amino on the 74th. day of treatment | 6 | — | + | — | + | +++ |
| Primaquine | 3 | + | ++ | — | + | — |
| Amino + Primaquine | 4 | — | — | — | — | — |

The histopathological findings are registered in Table II. We found the following picture among the untreated animals (control): *heart* — the parasitism of the cardiac muscle fibers was very marked in all mice except 1 which showed a moderate number of parasites (Fig. 1). The inflammatory reaction was not intense, being focal and slight, or diffuse and moderate in degree. It was made up of lymphocytes, monocytes and histiocytes with rare fibroblasts and neutrophil leucocytes. The areas with more marked cellular infiltration showed dissociation and destruction of the myocardial fibers; *skeletal muscles* — the parasitism of the muscle fibers was of slight (2 mice) or moderate (3 mice) degree and the inflammatory reaction was inconspicuous or not observed; *kidneys* — only nuclear pyknosis of a few cells of the convoluted tubules was observed.

Dimethyladenine treated animals: *heart* — the same findings as in the control animals, but with slight parasitism in 2 mice, 1 dying on the 26$^{th}$ day after the inoculation and the other sacrificed on the 74$^{th}$ day. In these 2 animals the inflammatory reaction presented more numerous fibroblasts; *skeletal muscles* — the parasitism was in most cases of slight degree. However, in 1 case it was moderate and in another marked. Inflammatory reaction was similar to control animals, being, however, less frequent and in moderate degree; *kidneys* — convoluted tubules with the same nuclear pyknosis as seen in the control group, being more frequent, however, in 4 animals.

Aminonucleoside of stylomycin: we found among the 3 animals sacrificed on the 14$^{th}$ day after the *T. cruzi* inoculation: the parasitism and the inflammatory reaction in the heart were less intense than in controls; skeletal muscles did not present parasites and the kidneys showed no changes. The animals sacrificed on the 74$^{th}$ day after the *T. cruzi* inoculation presented: *heart* — only in 1 of the 6 mice rare parasites were found in the myocardial fibers. A slight focal inflammatory reaction was present in 4 mice and failed to be observed in 2 (Fig. 2). This reaction was constituted by rare and small foci of lymphocytes and monocytes

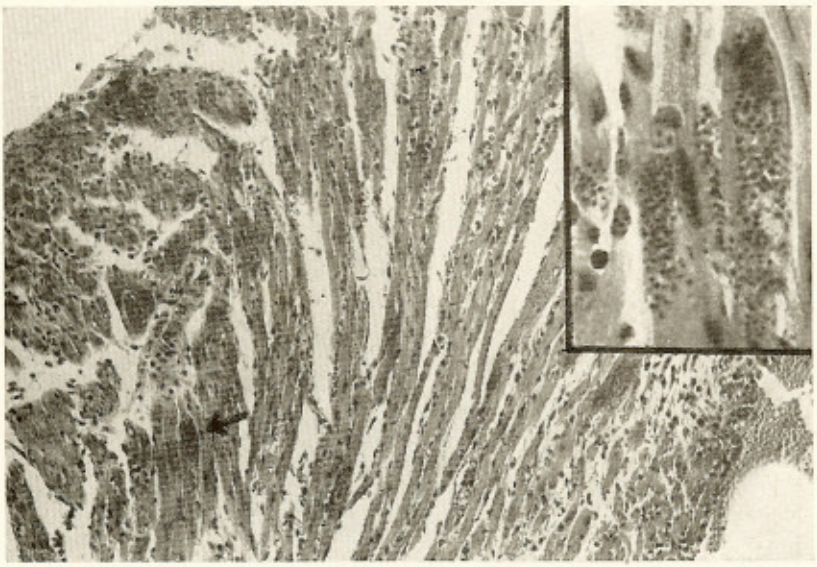

*Fig. 1 — Control animal (15 days after the* Trypanosoma *inoculation). Notice the high parasitism of the myocardial fibers. Hematoxylin and eosin stain. ×136. At the right upper corner detail of the myocardial fibers seen near the left lower corner (arrow). ×544.*

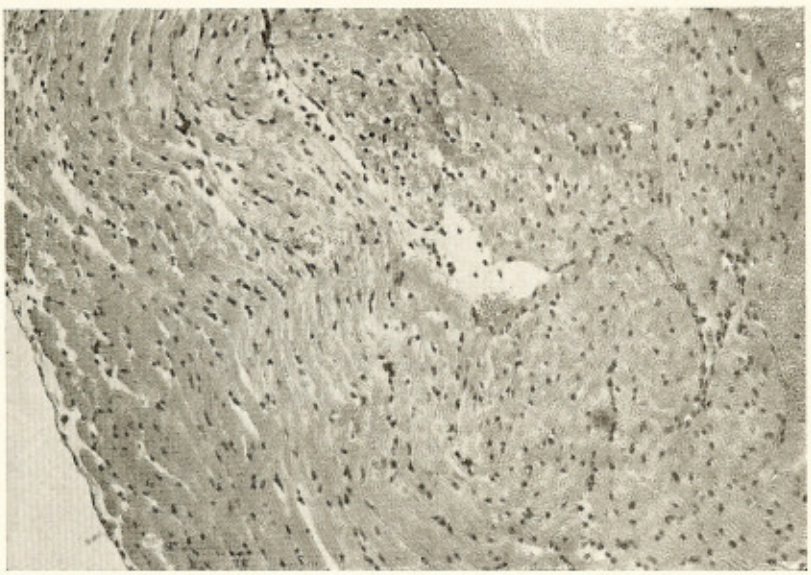

*Fig. 2 — Animal treated with aminonucleoside (7½ days after the* Trypanosoma *inoculation). Section at about the same level of the Fig. 1 showing a normal picture of the myocardium. Hematoxylin and eosin stain. ×136.*

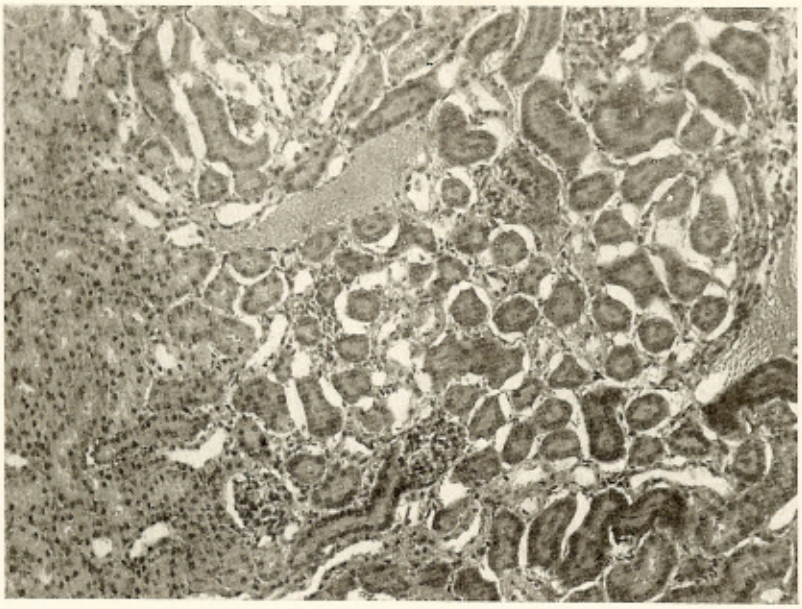

*Fig. 3 — Animal of the same group of the Fig. 2. At right, atrophy of the renal tubules with interstitial fibroblastic proliferation. Hematoxylin and eosin stain. ×148.*

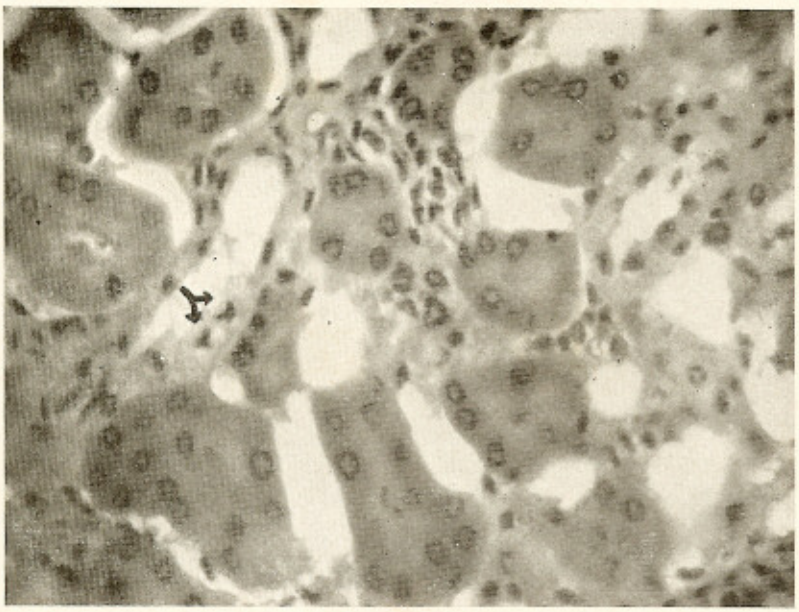

*Fig. 4 — Detail of the anterior figure, showing nuclear pyknosis of the tubular cells at left (×) and interstitial fibroblastic proliferation. ×592.*

scattered in the myocardium, and it was more conspicuous in the case with parasites. In one of the 2 cases without inflammation the myocardium presented a few small scars; *skeletal muscles* — no parasitism, and the inflammatory reaction as in the heart; *kidneys* — the cortex was diffusely loosened and edematous with widening of the interstitial spaces and fibroblastic proliferation. This picture was of moderate degree in 4 animals and slight in 2. A few cortical tubules presented necrosis of single cells and nuclear pyknosis of other elements (Figs. 3, 4). Also other elements of the proximal and distal convoluted tubules and Henle's loop showed basophilic cytoplasm and normal nuclei. The cells of the collecting tubules presented no changes. Rare epithelial cells and acidophilic networks in the tubular lumina were seen. Small foci of round cells seen in 2 mice only. No thickening of the basement membrane of the glomerular capillaries was observed.

Primaquine treated animals: *heart* — rare parasitism of the myocardial fibers in 2 mice and none in the others: slight focal inflammatory reaction of round cells; *skeletal muscles* — in 2 examined mice an inflammatory reaction as in the heart was observed, but without parasites; *kidneys* — no changes could be found.

Animals treated with primaquine plus aminonucleoside: *heart* — no parasites were found; rare and small lymphocytic foci were observed in 1 animal only; *skeletal muscles* — no changes; *kidneys* — the cells of the convoluted tubules presented rare nuclear pyknosis, which was more frequent in 1 mouse. Thickening of the basement membrane of the glomerular capillaries was not seen.

## DISCUSSION

There is a widespread opinion among the workers dealing in Chagas disease, that the failure to find an efficient chemotherapy seems to be due to the high resistance of its ethiologic agent when in the intracellular stage of its evolutive cycle. This situation is really unfortunate, because the parasite multiplication forms are just the intracellular leishmania, that become of course, the target which the chemotherapist aims to hit. The association of a drug whose site of action seems to be on the intracellular leishmania (aminonucleoside) with another drug remarkably effective against the extracellular parasites (primaquine) seems to be very promising.

The histologic examination of the heart and skeletal muscles (the tissues with greater parasitism) confirmed the parasitologic findings. All the animals, but one, treated with the aminonucleoside showed, on the 74th day after the *Trypanosoma* inoculation, no leishmania in the heart and skeletal muscles. At this time the inflammatory reaction in these organs was slight or absent. A more conspicuous action of the treatment was observed in the animals receiving aminonucleoside and primaquine in which no parasites and generally no inflammatory reaction were present.

The kidneys of the animals receiving the aminonucleoside for a longer time (74 days) presented necrotic changes of the tubular cells and interstitial fibroblastic proliferation. This effect, possibly due to the action of the drug on the renal tubules, was not observed in the animals sacrificed earlier (14-31 days). The nuclear pyknosis found in a few tubular cells of the control animals and in animals treated with dimethyladenine is probably due to the trypanosomiasis itself. A thickening of the glomerular basement membrane as observed by others (FRENK *et al.*[4]), was not seen in any group of animals.

No doubt exists as to trying the treatment of human Chagas disease with primaquine. The toxicity of this drug is very well known, while that of the aminonucleoside of stylomycin is not known in relation to man. However, while waiting for a really effective treatment for Chagas disease it seems of interest to try the association of drugs we report in this paper, once a careful criterion for toxicity is followed. Unpublished results from this laboratory (J. P.

M. PEREIRA, personnal communication) have shown very little, if any, toxicity of the aminonucleoside of stylomycin to bone marrow. However, the renal toxicity of the drug to mice and rats requires careful control when assayed in man, especially if the treatment is prolonged.

The ineffectiveness of the $N_6$ diethyl analogue of stylomycin seems to indicate the essentiality of the methyl group in $N_6$ of the purine ring. The inefficacy of $N_6$ dimethyladenine is a further indication of the role of the pentose amine moiety of the molecule in the activity of the drug.

RESUMO

*Síntese de nucleotídio e polinucleotídio em "Trypanosoma cruzi". V. Efeitos da primaquina, derivados e análogos da estilomicina, sôbre camundongos infectados experimentalmente.*

Camundongos jovens foram inoculados com uma cêpa virulenta de *Trypanosoma cruzi* e tratados com primaquina, isoladamente ou em associação com o aminonucleosídio da estilomicina, o seu $N_6$ dietil derivado e também com $N_6$ dimetiladenina. Os seguintes resultados foram observados nos animais tratados com primaquina e aminonucleosídio da estilomicina:

1. Sobrevivência de todos os animais tratados enquanto ocorreu 100% de morte entre os animais não tratados.

2. Número insignificante ou ausência de flagelados no sangue circulante.

3. Efeitos histopatológicos nìtidamente favoráveis sôbre os músculos cardíacos e esqueléticos e nenhum sinal de toxicidade da droga para o rim, quando o tratamento com o antipurínico não era demasiadamente prolongado.

4. Quando as drogas eram administradas isoladamente os efeitos acima enumerados eram menos pronunciados.

5. $N_6$ dietil análogo do aminonucleosídio bem como a $N_6$ dimetiladenina se mostraram pràticamente sem ação na infecção experimental de camundongos pelo *Trypanosoma cruzi*.

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