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"IN VITRO" EFFECT OF THE AMINONUCLEOSIDE OF STYLOMYCIN AND DIMETHYL-ADENINE AGAINST *TOXOPLASMA GONDII*

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SUMMARY

The aminonucleoside of Stylomycin and the purine base of the antibiotic, dimethyl-adenine, show considerable toxic activity against *Toxoplasma gondii* in tissue culture. The analogs inhibit the reproduction of the parasite and determine total disintegration of the intracellular parasites without degeneration of the host cells. No reversion of the drug action was obtained by the use of adenine, adenosine and desoxyadenosine.

INTRODUCTION

The action of the aminonucleoside of Stylomycin against *Trypanosoma cruzi* has been recently described, by FERNANDES *et al.*[4, 15]. The drug has an inhibitory effect on the incorporation of adenine into the nucleic acid of cultural forms of the parasite, interferes with the mitotic process and determines degeneration of its intracellular leishmanial stages in tissue culture.

On the other hand, the activity of Stylomycin had been demonstrated some years ago against both *T. cruzi* and *Toxoplasma gondii* infections in mice (SONNTAG & KLOETZEL [16], PIZZI *et al.*[14], CHRISTEN & THIERMANN [1], EYLES & COLEMAN [3]). This analogy suggested to us the idea of studying the effect of the aminonucleoside of Stylomycin on *Toxoplasma*. Our investigations were prompted by the assumption that the aminonucleoside fraction of the antibiotic molecule might be responsible for the antitoxoplasmic activity of the drug. Apparently, this is the case in trypanosomiasis (FERNANDES & CASTELLANI [4], HEWITT *et al.*[7], TOBIE & HIGHMAN [17]).

Results obtained with the aminonucleoside on *Toxoplasma* infected tissue cultures are henceforth presented.

MATERIAL AND METHODS

The experiments were performed on HeLa cells cultures and trypsinized calf kidney tissue, both maintained in stationary test tubes with flying coverslips. The nutrient medium for HeLa cells consisted of Hanks' balanced solution with 0.25% lactoalbumin hydrolysate (Nutritional Biochemical Corp.) and 10% sheep serum inactived at 56°C for 30 minutes. For trypsinized calf kidney, calf serum was used instead of sheep's. Penicillin and Streptomycin were added in final concentrations of 200 U.O./ml and 0.05 mg/ml each. The nutrient medium was adjusted to pH 7.2-7.4 with sodium bicarbonate and distributed in amounts of 1.5 ml for each culture tube.

The aminonucleoside of Stylomycin * and the whole antibiotic were supplied by Lederle Lab. Division, American Cyanamid

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* Stylomycin is the trade mark of the antibiotic formely named Achromycin and afterwards Puromycin (Lederle Lab. Div., American Cyanamid Co.).

Co. Dimethyl-adenine was obtained through acid hydrolysis (1N-HCl) of the aminonucleoside in boiling water bath for 1 hour. In some experiments we tried to counteract the effect of the aminonucleoside by using adenine (Sigma), adenosine and desoxyadenosine (Nutritional Biological Corp.). All drugs were prepared in concentrated solutions in Hanks' and sterilized in Seitz filters.

By means of preliminary tests, using non-infected tissue cultures, the toxic levels of the drugs were established.

The parasite used was the "N" strain of *Toxoplasma gondii*, originally isolated by NOBREGA [13] and maintained in our laboratory through successive transfers every 2-3 days, in mice.

The tissue cultures were infected, after 3-7 days incubation at 37°C, with a saline suspension of peritoneal exudate taken from mice with a 2-day infection. To each culture tube was added 0.1-0.2 ml of exudate suspension, an amount which was made to contain 4 to 6×10^6 extracellular toxoplasmas, according to prior countings in a Neubauer haemocytometer. The tubes were then incubated for another 6-8 hours, after which the nutrient medium was substituted for a new one containing the drug (or drugs) to be tested. Sometimes the drug was added together with the parasites.

The antitoxoplasmic activity of the drug was estimated, through microscopic examination of the coverslips stained by Giemsa, on the basis of: 1) proportion of infected to uninfected cells; 2) number of parasites within infected cells; 3) morphological aspect of parasites and host cells. For each test, equal number (3 to 6) of control (untreated) and treated preparations, were used.

RESULTS

With a 0.03 to 0.05 mM concentration, which has been used in almost all the experiments, the aminonucleoside of Stylomycin did not seem to harm the tissue cells, even after 5 days of contact. In higher concentrations it causes some inhibition of the mitotic process, as indicated by the accumulation of metaphase figures. A total degeneration of the tissue cells occurred after 6 days of contact with the drug when this was used at a 0.6 mM concentration.

Stylomycin is much more toxic for the cells than its aminonucleoside moiety. Even at 0.03 mM, it causes very rapid degeneration of the cells. This fact prevented us from testing the antitoxoplasmic activity of the whole antibiotic on tissue cultures. Since dimethyl-adenine is less toxic than the aminonucleoside we compared both drugs in equal molar concentrations of 0.03 mM.

1. *Effect of the aminonucleoside of Stylomycin against "Toxoplasma"* — With 0.03 to 0.05 mM concentrations, the aminonucleoside markedly inhibited reproduction of the intracellular parasites and caused their progressive degeneration. The first morphological signs of such action appeared after 2 days with a swelling of the parasites and the appearance of many eosinophilic granules in their cytoplasma. Around the 60th hour, we observed many parasites with a picnotic nucleus. Later, the nuclei appeared split in fragments (karyorrhexis) (Figs. 5 and 6). At this time, there was a significant difference in the averages of parasites per parasitized cells, between treated and control cultures (Table I). After 4 days practically all parasites found in treated cultures were degenerate (Figs. 7 and 8). In many, the degree of disintegration was such that they would not be recognized as toxoplasmas if it were not by comparison with a few neighbouring forms showing earlier stages of degeneration. Because of this fact it was sometimes difficult to count the parasites. However the contrast in number between the treated and the control cultures was so obvious that a precise count was superfluous. Table I compares the treated with the control cultures. In the treated cultures the parasites stayed at the

TABLE I

Effect of the aminonucleoside of Stylomycin against *Toxoplasma* in HeLa cells. (final concentration of the drug — 0.03 mM)

Group	*Time of fixation after infection (in hours)*	*Number of slides*	*Number of parasitized cells counted*	*Frequency of the indicated nuber of toxoplasmas per cell*									
				1-2		3-4		5-10		11-20		> 20	
				N	%	N	%	N	%	N	%	N	%
Experimental	60	3	174	106	61.0	59	34.0	7	4.0	2	1.0	—	—
(Treated)	120	3	161	100	62.0	48	30.0	13	8.0	—	—	—	—
Control	60	2	103	33	32.0	22	21.5	29	28.0	14	13.5	5	5.0
(Untreated)	120	4	245	12	5.0	15	6.0	18	7.0	51	21.0	149	61.0

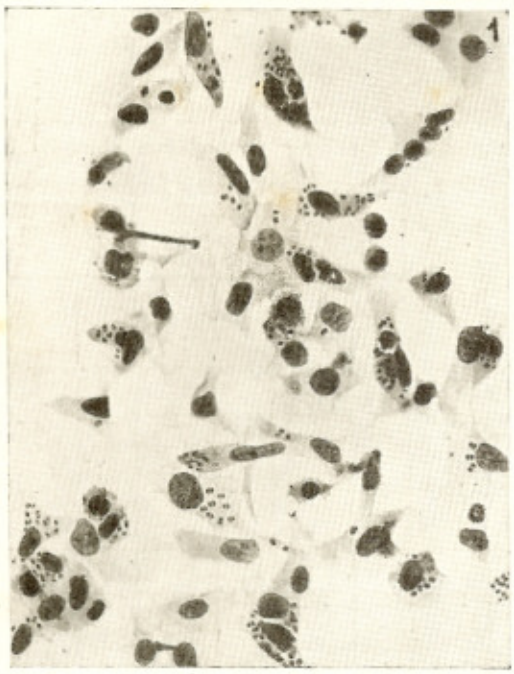
same quantitative levels from the 60th to the 120th hour after addition of the drug. However in the control cultures there was a marked increase in number (Figs. 1 to 4).

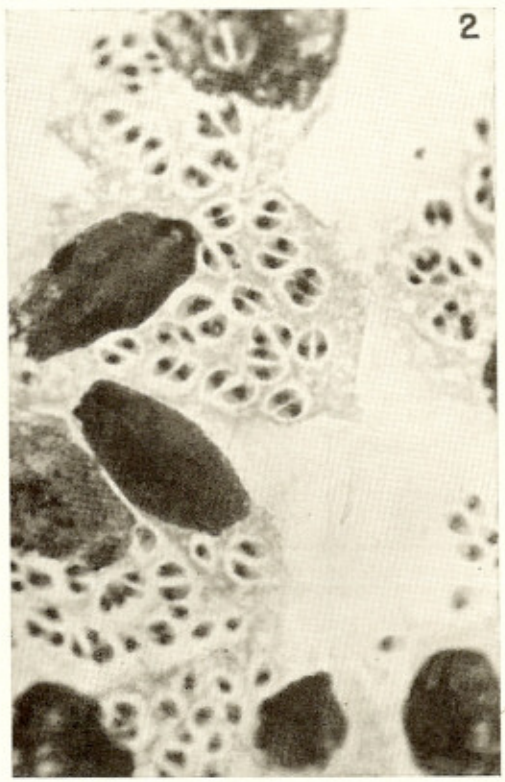
Apparently the degree of degeneration presented by the toxoplasmas was related to the number inside a given cell. In other words, the heavier the parasite load within a cell, the more intense the degeneration of the parasites. Moreover, it seems that, if not heavily parasitized, the host cells are able to survive the degenerative process suffered by the parasites. This phenomenon was observed with many cells of an entirely normal aspect, presenting only an eosinophilic area surrounding the disintegrating toxoplasmas. On the other hand, the cell itself degenerates if loaded with a large number of degenerating parasites.

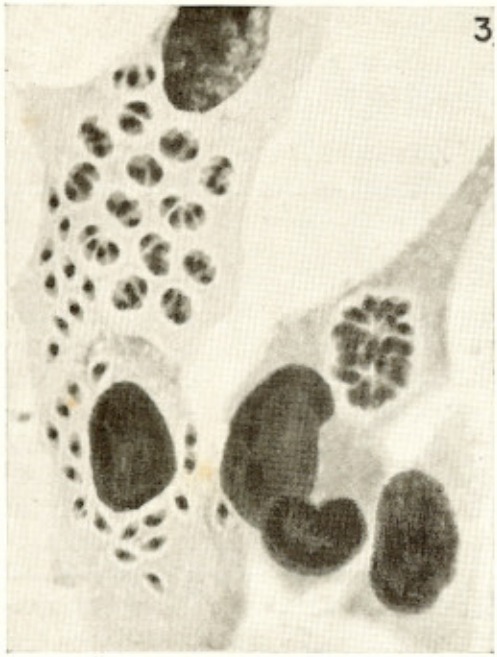
If both the aminonucleoside and the infective material were added together to the tissue cultures, many parasites were found within the tissue cells, 6 to 12 hours later. This seems to indicate that the drug does not interfere with the penetration of the parasite into the cells.

2. *The action of dimethyl-adenine against "Toxoplasma"* — In two experiments, dimethyl-adenine was added to the nutrient medium in final concentration of 0.03 mM. The purine base showed a remarkable antitoxoplasmic action in tissue culture, since, as the aminonucleoside, it inhibited the intracellular reproduction of the parasites and caused them to degenerate within a few days (Figs. 9 and 10). Although we could not compare the effect of both drugs in quantitative terms, we have the impression that the aminonucleoside is more active than its purine base, since in equal molar concentrations the degeneration of parasites occurs earlier with the aminonucleoside.

3. *Experiments on the reversion of the aminonucleoside activity* — In other experiments we tried to counteract the effect of







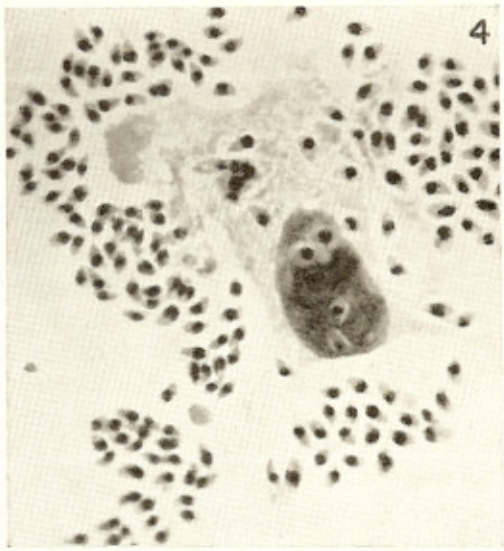
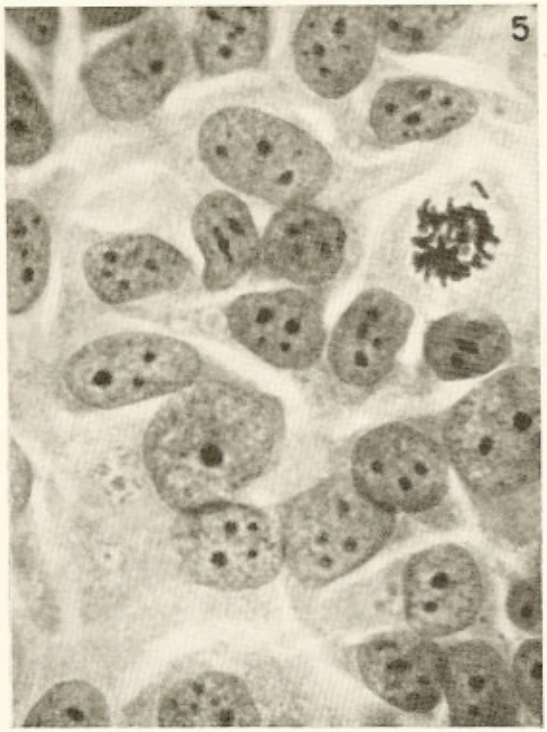
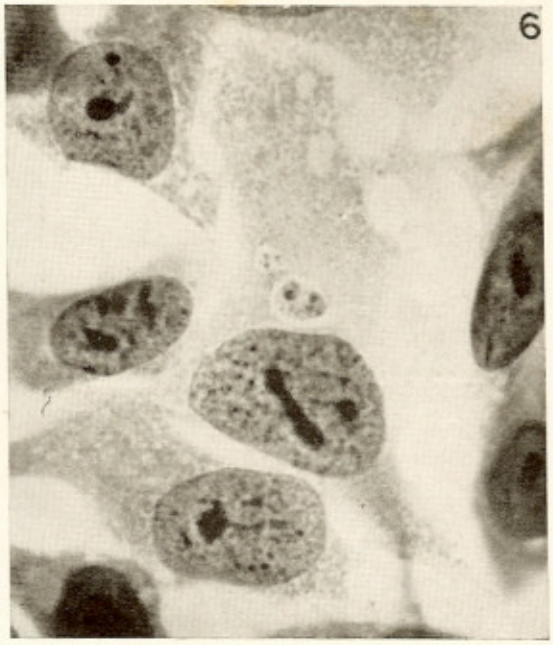


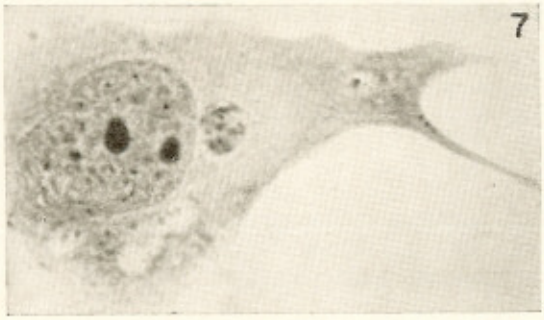
Fig. 1-4 — Tissue culture (HeLa cell) infected with Toxoplasma gondii, *in the 4th day of infection. Fig. 1 — Low power (×300) showing almost all cells parasitized. Fig. 2 & 3 — Parasites loading the cytoplasm of culture cells (×1,500); notice normal structure of parasites. Fig. 4 — Disintegration of parasitized cell with liberation of intracellular toxoplasmas (×1,500).*

the aminonucleoside of Stylomycin against *Toxoplasma*, by adding adenine, adenosine or desoxyadenosine, together with the aminonucleoside, to the nutrient medium of infected cultures. No reversal of the toxic effect against the parasite was demonstrable when these metabolites were used in a 10-fold ratio with the aminonucleoside.

For the purpose of discrimination one experiment was performed using a 30-fold ratio, i.e., 0.03 mM of aminonucleoside and 0.9 mM of adenine. The controls showed







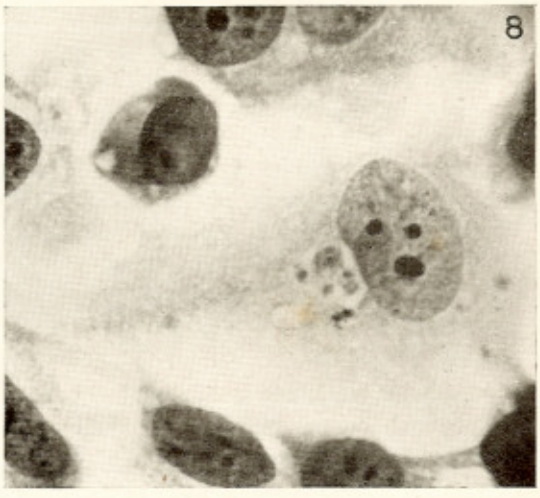
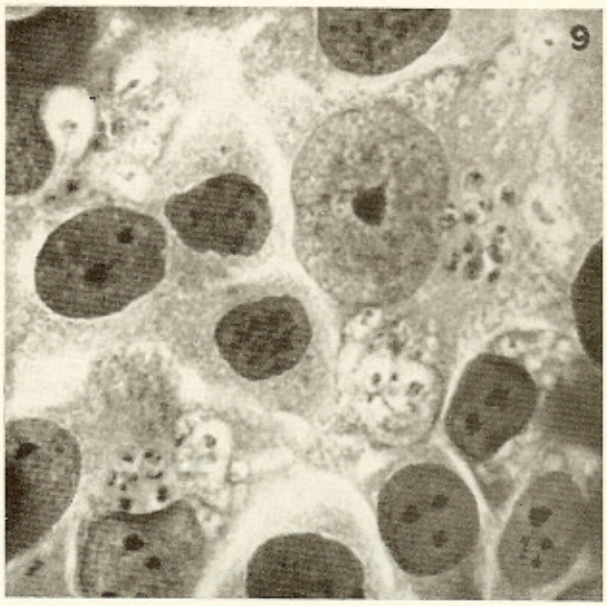


Fig. 5-8 — Effect of the aminonucleoside of Stylomycin against Toxoplasma gondii. *Fig. 5 — Parasitized tissue culture showing degenerated parasites (low, left) after 2 days of drug action (×1,000). Fig. 6 — Same, showing parasites in karyorrhexis (×1,500). Fig. 7 & 8 Parasitized cells after 4 days of drug action, showing disintegration of toxoplasmas (×1,500).*

a toxic ativity of adenine alone against the tissue cells, but even so, the parasites found within normal cells of adenine-control culture tubes, were abundant and structurally normal after 4 days of contact. However in the adenine-aminonucleoside preparations the degeneration of the parasites progressed in the same way as observed in the absence of the metabolite.



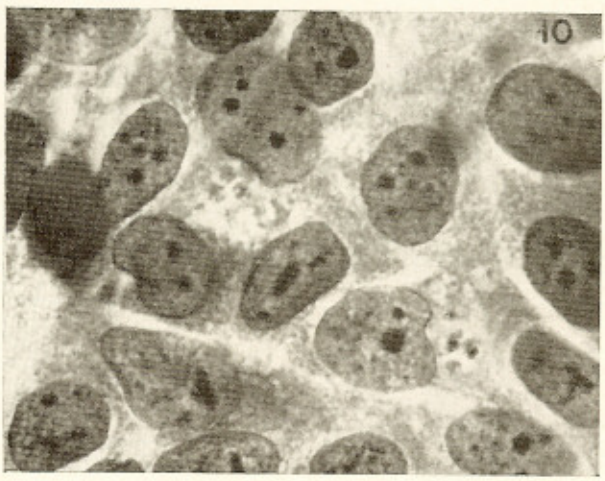


Fig. 9-10 — Effect of dimethyl-adenine against Toxoplasma gondii. *Fig. 9 & 10 — Parasitized tissue culture showing advanced degeneration of toxoplasmas after 4 days of drug action (×1,500).*

DISCUSSION

The results of our experiments indicate that the aminonucleoside moiety of Stylomycin is responsible for the antitoxoplasmic activity. Dimethyl-adenine, the purine base of the antibiotic, also has a toxic effect against *Toxoplasma* but it is of a lower degree. This difference in effectiveness between aminonucleoside and dimethyl-adenine was previously observed with *Trypanosoma cruzi* (Fernandes, Moraes & Silva, unpublished data).

As we can see in the review by Hutchings [8], the activity of the intact molecule of the antibiotic against bacteria, *Entamoeba histolytica* and some worms, is not presented by the aminonucleoside moiety, but, on the other hand, this is, on a molar basis, more active against trypanosomes and the mammary adenocarcinoma of the C_3H mouse. The activity against *Trypanosoma equiperdum* may be altered by structural changes in the dimethyl radicals of the 6-position of the purine ring.

Both the aminonucleoside and the dimethyl-adenine base seem to be active only during the reproduction phase of the parasite. Inhibition of parasite division was marked, even before any degenerative phenomena were apparent. There is also interference with the mitotic process, as indicated by the fact that we have often seen bi-nucleated parasites, although this occurrence is less frequent than in the case of *Trypanosoma cruzi* (Silva *et al.*[15]).

On the other hand, the drug seems to be innoxious to extracellular parasites and does not inhibit their penetration into the cells. When parasites were added to cultures with drug-containing nutrient medium, no dif-

ferences could be found in the proportion os parasitized to free cells between control and treated cultures, 6 to 12 hours after contact with the drug.

The toxicity of the aminonucleoside for HeLa cell is higher than for embryonic chicken cells (SILVA *et al.*[15]), as it would be expected from the known activity of the drug against some tumors (TROY *et al.*[apud 8]). However, we do not believe that the demonstrated effect against the parasite is the indirect result of the drug's action against the cells, because we have considered only the degeneration of toxoplasmas harboured by cells with a normal aspect. Also, we have noticed that the disintegration of parasites is not usually accompanied by the degeneration of host cells, unless the number of intracellular parasites is very large. Furthermore we followed many infected cultures for more than 10 days after the drug had been removed and we could observe a good cellular growth with the absence of parasites. In contrast, the control infected cultures degenerated in a few days, due to parasitic action. Finally in non cancerous cells, namely trypsinized calf kidney tissue, the same effect of the drug against the parasite was demonstrated.

From the known data on trypanosomiasis we could expect the drug to act as antagonist of purine nucleotide metabolism. No conclusive data concerning the mechanism of anti-toxoplasmic activity could be evaluated from our experiments on reversion, but the toxic effect on dimethyl-adenine agrees with this hypothesis. Actually, if the purine base is normally found in a little proportion in *Aerobacter aerogenes* and *Escherichia coli's* RNA (LITTLEFIELD & DUNN[11]) it seems reasonable to assume that it can be abnormally metabolized by other organisms.

Since 2-6-diaminopurine has some inhibitory effect on the reproduction of *Toxoplasma* (COOK[2]) and dimethyl-adenine has a toxic effect against the parasite, the search for other purine analogs with chemotherapeutic value against the parasite might be rewarding. A positive result in this field would be important because previously the most effective drugs against the parasite — sulfonamides and pyrimethamine — act on the same metabolic sequence (FRENKEL & HITCHINGS[5]), i.e., antagonizing the synthesis of the formil-donor coenzyme, which is so important in many biochemical reactions, particularlw in the "de novo" biosynthesis of purine nucleotide (GREENBERG & JAENICKE[6]). The possibility of acting on another metabolic process of the parasite that is, on the "salvation pathway" for nucleotide synthesis (KORNBERG[9]) or purine containing coenzymes synthesis, would be of great interest for the treatment of toxoplasmosis. In the chemotherapy of experimental leukemia and cancer, for example, and in the action against several bacteria, very favorable results have been obtained with the association of anti-folic and purine analogs (NICHOL[12], LACEY[10]).

RESUMO

Ação "in vitro" do aminonucleosídio da Estilomicina e da dimetiladenina contra o "Toxoplasma gondii".

O autor estuda a ação do aminonucleosídio da Estilomicina e da dimetil-adenina sôbre o *Toxoplasma gondii* em cultura de tecido. Ambas as drogas demonstraram ação tóxica intensa sôbre o parasita, principalmente o aminonucleosídio, avaliada pela inibição da reprodução e degeneração progressiva até lise total dos parasitas intracelulares. Nas concentrações até 0,05 mM ativas contra o *Toxoplasma*, não houve degeneração das células da cultura.

Os resultados parecem indicar que a fração aminonucleosídio é a fração ativa do antibiótico contra o *Toxoplasma*, mas não se conseguiu reversão da ação tóxica contra o parasita, pelo emprêgo de adenina, adenosina e desoxiadenosina, nada podendo se adiantar, portanto, quanto ao mecanismo de ação dêste análogo de purina.

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