Revista Latinoamericana de Microbiología **(2001) 43:1-6**
Asociación Latinoamericana de Microbiología

Trypanocidal Activity of 4 Isopropyl Salicylaldehyde and 4-Isopropyl Salicylic Acid on *Trypanosoma cruzi*

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ABSTRACT. We synthesized and study the possible trypanocidal activity of 4-isopropyl salicylaldehyde and 4-isopropyl salicylic acid, these compounds were chemical derivatives of gossypol, a drug that inhibits the growth of *T. cruzi* in culture. These derivatives were tested in two *T. cruzi* strains with different *in vitro* susceptibility to benznidazole and nifurtimox, used as a reference drugs. It was found that they were better inhibitors of *T. cruzi* α-hydroxyacid dehydrogenase than gossypol, in both strains. The *in vitro* and *in vivo* pharmacological tests were performed and in both test, the gossypol derivatives showed a higher and better trypanocidal effect than gossypol, and a higher and much better trypanocidal effect than nifurtimox and benznidazole in the two studied *T. cruzi* strains. The trypanocidal effect was higher in the NINOA strain than in the MIGUZ strain.

Key words. *Trypanosoma cruzi*, trypanocidal drugs, 4-isopropyl salicylaldehyde, 4-isopropyl salicylic acid.

RESUMEN. Se sintetizó y evaluó la posible actividad tripanomicida de dos compuestos, el 4-isopropil salicilaldeído y el ácido 4-isopropil salicílico, compuestos químicos derivados del gosipol, fármaco que inhibe el crecimiento de *T. cruzi* en medios de cultivo. Estos derivados fueron probados en dos cepas de *T. cruzi* con diferente susceptibilidad *in vitro* al benznidazol y nifurtimox, fármacos que fueron usados como referencia. Se encontró que los dos derivados son mejores inhibidores de la α-hidroxiácido deshidrogenasa de ambas cepas, que el gosipol. Tanto *in vitro* como *in vivo*, los derivados del gosipol mostraron un mejor y mayor efecto tripanomicida que el gosipol y un mejor y mayor efecto tripanomicida que el nifurtimox y el benznidazol en las dos cepas del parásito estudiadas. El efecto tripanomicida fue más alto en la cepa NINOA en comparación a la cepa MIGUZ.

Palabras clave. *Trypanosoma cruzi*, drogas tripanosomicidas, 4-isopropil salicilaldeído, ácido 4-isopropil salicílico.

INTRODUCTION

Since the 80's, it has been reported that some antispermatogenic drugs can be used also to inhibit the growth of trypanosomes, because there are several biochemical similarities between trypanosomes and spermatozoa.[13,15,24] They have one characteristic in common which make them different from most other mammalian cells and is the presence of a flagellum which is continuously moving with high spend of energy. The flagellum is essential for moving forward either to obtain nutrients like in trypanosomes or to reach the ovule like in spermatozoa. In addition, the parasites tend to replicate faster than mammalian cells increasing even more their need for energy. According to this, it has been proposed that interfering the energy metabolism of the parasite it can be reduce the trypanosomal infection.[3]

Investigations carried out by Blanco and his group[8,9] demonstrated that *Trypanosoma cruzi*, the flagellate parasite that causes Chagas' disease, possesses an enzyme, α–hydroxyacid dehydrogenase, very similar to LDHx (EC 1.1.1.27, L-lactate:NAD oxidoreductase) of mammalian spermatozoa.[13] Total extracts of cultured epimastigotes of *T. cruzi* posses a NAD-linked oxidoreductase whose catalytic properties resemble those of mouse LDHx and rat.[4] This enzyme was designated α–hydroxyacid dehydrogenase (HADH).[12] On account of the similarities between HADH and LDHx, it was assumed that these enzymes are metabolically and functionally homologous. Thus, α–hydroxyacid dehydrogenase like LDHx may be also integrated in the metabolic pathways designed to supply energy for the motility and survival of *T. cruzi*.

Evidences has been accumulated during the last 30 years indicating that gossypol, a polyphenolic compound present in the cotton plant seeds, acts as an antifertility agent in man and in males of several species of mammals. Therefore, it has been used as a male contraceptive agent.[14,26] It has been found that this compound exerts its antifertility

effect because inhibits the lactate dehydrogenase isozyme X (LDHx or C_4) that is implicate in the regulation of glycolysis, the unique metabolic process that provides energy for motility and survival of the male gamete.[19]

It has been reported that gossypol inhibits the growth of american[4,15,24] and african[10] trypanosomes in culture because it is a powerful inhibitor of their energetic metabolism. But it has been found that gossypol is an unspecific inhibitor because competes with the coenzyme NAD, and therefore inhibits most of the dehydrogenases that use this coenzyme.[13]

In previous studies, we synthesized and characterized two gossypol derivatives, 4-isopropyl salicylaldehyde and 4-isopropyl salicylic acid (Fig. 1). Both of them were competitive inhibitors of LDHx[21] and therefore we decided to investigate the effect of these compounds on *T. cruzi* α–hydroxyacid dehydrogenase and their possible *in vivo* and *in vitro* trypanocidal activity on this parasite.

MATERIAL AND METHODS

Chemicals. All reagents used were purchased from Sigma Chemical Co., St Louis, MO, USA and were of the maximum quality available.

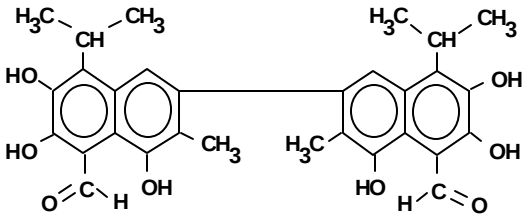
Synthesis of 4-isopropyl salicylaldehyde. This compound was synthesized by the reaction of 3-isopropylphenol with chloroform in the presence of a strong base like NaOH using the Reimer-Tiemann reaction.[25] The obtained final oily residue was fractionated under reduced pressure, collecting the 4-isopropyl salycilaldehyde (a yellowish liquid) as a fraction of bp 91-92°C/ 4 mm Hg. The chemical structure of this compound was confirmed by spectroscopy.[21]

Synthesis of 4-isopropyl salicylic acid. The 4-isopropyl salicylaldehyde was transformed into 4-isopropyl salicylic acid by the Cannizaro reaction,[25] giving colorless crystals, mp 73-75°C of pure 4-isopropyl salicylic acid. The chemical structure of this compound was confirmed by spectroscopy.[21]

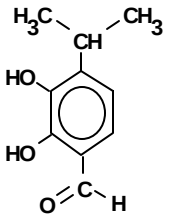
***T. cruzi* strains.** The *T. cruzi* strains were isolated through xenodiagnostic and haemoculture in LIT media from chronic chagasic patients. We employed two *T. cruzi* strains selected for their susceptibility to nifurtimox and benznidazole *in vitro* (the only available drugs in the market for the treatment of Chagas' disease): a strain resistant to benznidazole and nifurtimox (MIGUZ strain) and the other one partially susceptible to benznidazole (20%) and resistant to nifurtimox (NINOA strain).

α-hydroxyacid dehydrogenase assay. We used this method for both, the epimastigotes and trypomastigotes of *T. cruzi* extracts obtained 7 days after haemoculture using α-ketoisocaproate as a substrate according to a published procedure.[8]

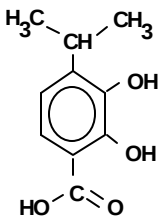
Determination of the trypanocidal activity *in vitro* (method slightly modified by Blanco[3]). NINOA and MIGUZ strains were cultured in liquid medium at 28°C. The



Gossypol



4-Isopropyl Salicylaldehyde



4-Isopropyl Salicylic Acid

Fig. 1. Structural relationships between gossypol and 4-isopropyl salicylaldehyde and 4-isopropyl salicylic acid.

epimastigotes were harvested on the 7th day during the exponential growth of the trypanosomes. The medium was centrifuged at 3000 x *g* for 15 min and the pellet of parasites were washed and resuspended in about 20 vol of the same medium. The pellet was dispersed in the liquid medium to a final concentration of 1 X 10^6 trypanosomes/ml. All the drugs were dissolved in ethanol. To an aliquot of the trypanosomal suspension the drugs were added to obtain a 10 µM final concentration. The control used, contained ethanol in the same proportion utilized to dissolved the drugs. All samples were incubated at 30°C. Observations and counts of the epimastigotes were carried out in a Neubauer haemacytometer at different times of incubation, we assumed that immobilized organisms were died. The same procedure was followed if blood trypomastigotes were employed, obtained by DEAE-cellullose chromatography.

Determination of the trypanocidal activity *in vivo*. This procedure was carried out according to Filardi and Brener (1984) with little modifications: Male albino mice CD1 strain, 18-20 g, were inoculated intraperitoneally with 1 X 10^4 blood trypomastigotes. At the peak of the parasitemia (38 days with MIGUZ and 46 days with NINOA strains) a single dose (500 mg/Kg) of the drug to be tested was given by oral route. The number of circulating bloodstream *T. cruzi* forms were detected according to Brener (1962), before and 2, 4, 6, 8 h after drug administration. Untreated mice similarly inoculated were used as controls. The percentage of reduction of the parasitemia was calcu-

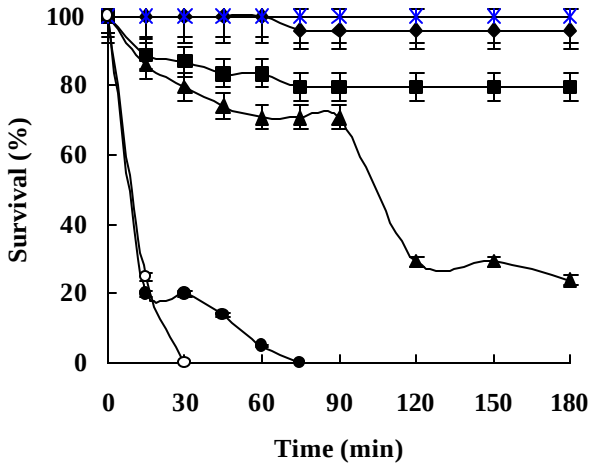


Fig. 2. *In vitro* trypanocidal activity of several drugs on *T. cruzi* epimastigotes of NINOA strain. The drugs were added at 10 µM (final concentration) and their effects were evaluated as described in material and methods. Æ, control; ♦, nifurtimox; , benznidazol; , gossypol; , 4-isopropyl salicylaldehyde; O, 4-isopropyl salicylic acid.

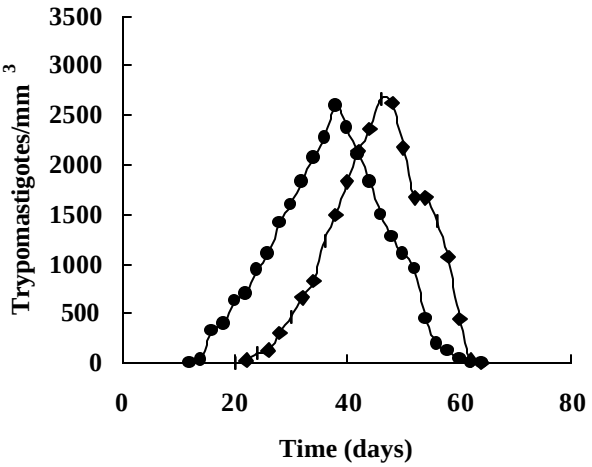


Fig. 4. Mice parasitemia of two *Trypanosoma cruzi* strains. •, MIGUZ strain and ♦, NINOA strain. The parasitemia was measured as indicated in material and methods.

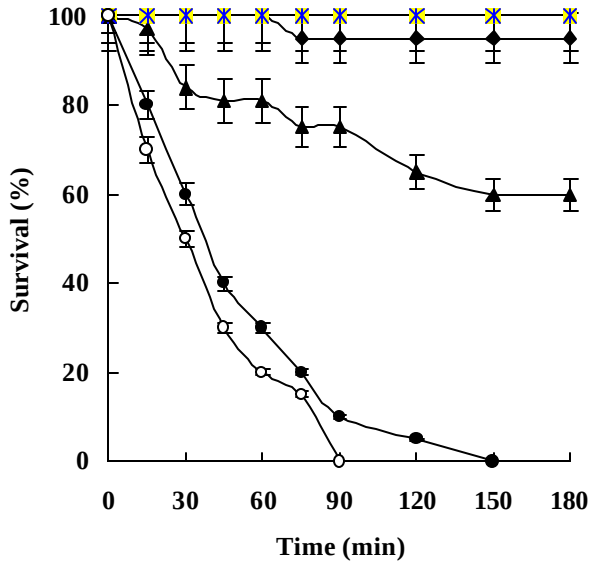


Fig. 3. *In vitro* trypanocidal activity of several drugs on *T. cruzi* epimastigotes of MIGUZ strain. The drugs were added at 10 µM (final concentration) and their effects were evaluated as described in material and methods. Æ, control; ♦, nifurtimox; , benznidazol; , gossypol; , 4-isopropyl salicylaldehyde; o, 4-isopropyl salicylic acid. Each point is the average of ten determinations.

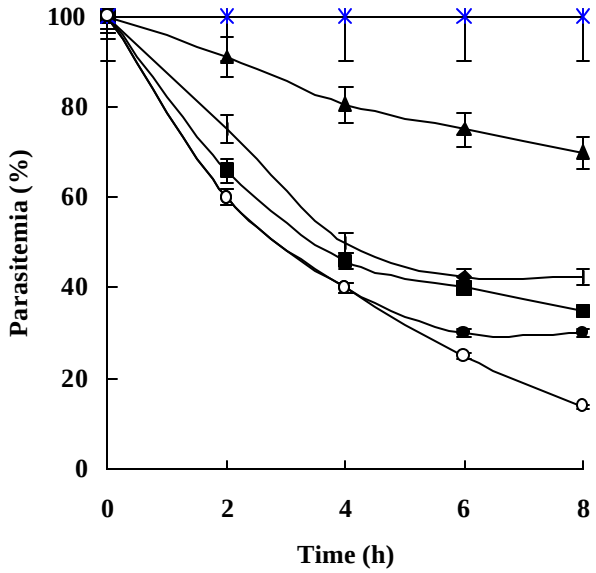


Fig. 5. *In vivo* Trypanocidal activity of several drugs on mice infected with NINOA strain. At the peak of parasitemia the drugs were given by oral route at 500 mg/Kg. Their trypanocidal effects were measured as described in material and methods. Æ, control; ♦, nifurtimox; , benznidazol; , gossypol; , 4-isopropyl salicylaldehyde; o, 4-isopropyl salicylic acid. Each point is the average of ten determinations.

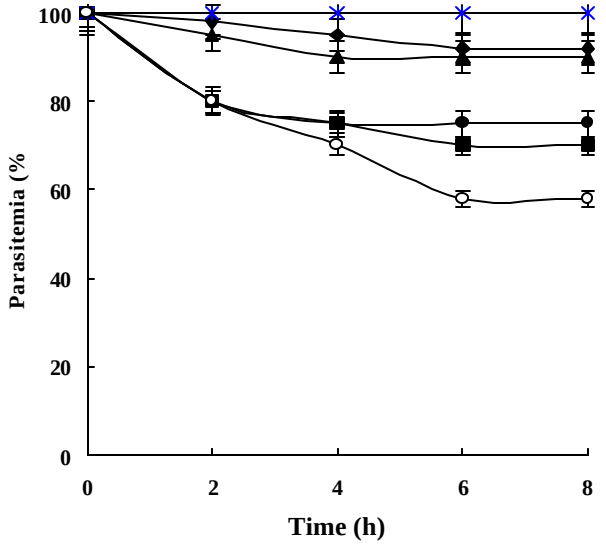


Fig. 6. *In vivo* trypanocidal activity of several drugs on mice infected with MIGUZ strain. At the peak of parasitemia the drugs were given by oral route at 500 mg/Kg. Their trypanocidal effects were measured as indicated in material and methods. Æ, control; ♦, nifurtimox; , benznidazol; , gossypol; , 4-isopropyl salicylaldehyde; o, 4-isopropyl salicylic acid. Each point is the average of ten determinations.

lated comparing the number of parasites obtained at each interval of time after drug administration, with that found before treatment.

RESULTS

The inhibitory effects of 4-isopropyl salicylaldehyde and 4-isopropyl salicylic acid on the activity of α hydroxyacid dehydrogenase it is shown on Table 1 and under the employed experimental conditions, both of this substances inhibited this enzyme better than gossypol. The IC_{50} for MIGUZ strain were 5, 2.3 y 15 µM for 4-isopropyl salicylaldehyde, 4-isopropyl salicylic acid and gossypol, respectively. The IC_{50} for NINOA strain were 1.5, 1.5 y 10 µM for 4-isopropyl salicylaldehyde, 4-isopropyl salicylic acid and gossypol, respectively.

Fig. 2 shows the *in vitro* trypanocidal activity of gossypol, nifurtimox, benznidazole and the synthesized compounds against epimastigotes of NINOA strain. It was observed that at concentration of 10 µM, 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde were the most effective of all. The 4-isopropyl salicylic acid and the 4-isopropyl salicylaldehyde kill all the parasites in 30 and 80 min respectively. Nifurtimox showed only a slightly trypanocidal activity against NINOA strain. The other drugs (gossypol and benznidazole) were not able to kill the trypanosomes completely, they were only partially effective.

Table 1. Inhibition of α-hydroxyacid dehydrogenase of two *T. cruzi* strains.

	IC_{50} (µM)	
Compound	MIGUZ Strain	NINOA Strain
Gossypol	15.0	10.0
4-isopropylsalicylaldehyde	5.0	1.5
4-isopropylsalicylic acid	2.3	1.5

T. cruzi α-hydroxyacid dehydrogenase activity was measured as indicated in material and methods. It was employed 0.15 mM α-ketoisocaproate in all determinations. IC_{50} is the inhibitor concentration causing a 50 percent inhibition of enzyme activity.

Fig. 3 shows the *in vitro* trypanocidal activity of gossypol, nifurtimox, benznidazole and the synthesized compounds against epimastigotes of MIGUZ strain. It is observed that at concentration of 10 µM, 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde were the most effective drugs of all. The 4-isopropyl salicylic acid and the 4-isopropyl salicylaldehyde kill all the parasites in 90 and 150 min, respectively. MIGUZ strain was resistant to benznidazol and almost resistant to nifurtimox, the other drug, gossypol, was not able to kill the trypanosomes completely. The same effects were seen when we used trypomastigotes instead of epimastigotes of both strain (results not shown).

The study of the trypanocidal effect of the drugs was initiated at the peak of the mice parasitemia, 38 days for MIGUZ strain and 46 days for NINOA strain (Fig. 4), with a single dose (500 mg/Kg) of the drug to be tested administered by oral route (*in vivo* effect). The number of circulating bloodstream *T. cruzi* forms were detected before and 2, 4, 6, 8 h after drug administration. In this experiment both 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde showed a better trypanocidal effect than benznidazole, nifurtimox and gossypol because the parasitemia was reduced 90 and 70 percent, with 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde, respectively, 8 h after they administration. However, at the same time, the parasitemia was reduced 35, 60 and 65 percent by gossypol, nifurtimox and benznidazole respectively (Fig. 5).

Fig. 6 shows the effect of 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde on the parasitemia of mice in-

fected with *T. cruzi* MIGUZ strain (*in vivo* effect) comparatively with gossypol, nifurtimox and benznidazole. The 4-isopropyl salicylic acid reduced to 40 percent the parasitemia, showing a better trypanocidal effect than benznidazole, nifurtimox and gossypol, 8 h after administration. The trypanocidal effect value of 4-isopropyl salicylaldehyde was between the observed for benznidazole and that observed for gossypol.

DISCUSSION

American trypanosomiasis (Chagas' disease) its an endemic parasitic disease afflicting more than 20 million people in Latin America. Currently therapy is unsatisfactory because only two drugs are available for its treatment, benznidazole and nifurtimox. Both of this drugs are toxic and in prolonged treatments they can produce cancer.[27] These drugs cure only a very low percentage of chronic patients.[7] Natural resistance of some *T. cruzi* strains to these drugs was suggested as an important factor to explain the low rate of cure detected in chagasic patients.[2] Contradictory results have been observed by different authors in the treatment of patients using benzonidazole and nifurtimox in different phases of the infection. The efficacy of these drugs ranged from 33.3% to 81%.[2,7] For this reason, most of the chronic patients do not receive curative treatments and this represents an epidemiological problem because they are parasite reservoirs.[20] This disease remains essentially incurable due principally to a lack of profit incentive the pharmaceutical industry has had limited interest in developing new antichagasic drugs and any attempt for obtaining a new drug against *T. cruzi* is well come.

The glycolytic enzymes have been suggested as a target for anti-trypanosomatid drug design, because glycolysis provides virtually all the energy for the bloodstream form of trypanosomatids.[1] Then, when the trypanosomes are starved without glucose or incubated with an inhibitor of the plasma membrane glucose transporter, they die in a few minutes.[3] *T. cruzi* α–hydroxyacid dehydrogenase is an important glycolytic enzyme due to its participation in the energy metabolism of the parasite and therefore, it has been speculated that an inhibitor of this enzyme could reduce the motility and survival of this parasite. On the other hand, α–hydroxyacid dehydrogenase presents some catalytic similarities with LDHx from spermatozoa[7] and consequently LDHx inhibitors could be also behave like α–hydroxyacid dehydrogenase inhibitors. The 4-isopropyl salicylaldehyde and its corresponding acid (4-isopropyl salicylic acid) were inhibitors of the LDHx[21] and according to this we supposed that probably they could be also inhibitors of *T. cruzi* α–hydroxyacid dehydrogenase.

Using *T. cruzi* extracts of MIGUZ and NINOA strains we tested the above mentioned compounds and we found that 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde really inhibit the α–hydroxyacid dehydrogenase of both strains from *T. cruzi*. These substances were much better inhibitors than gossypol and with these results we confirmed ours supposition: that only a chemical part of the gossypol molecule could be responsible for the inhibition of *T. cruzi* α–hydroxyacid dehydrogenase and that LDHx inhibitors could be also α–hydroxyacid dehydrogenase inhibitors. We also found that 4-isopropyl salicylic acid was a better inhibitor of α–hydroxyacid dehydrogenase than 4-isopropyl salicylaldehyde.

When we tested these inhibitors of ahydroxyacid dehydrogenase on *T. cruzi*, we observed a great *in vitro* trypanocidal effect with these gossypol derivatives. These compounds showed a higher and better trypanocidal effect than gossypol, benznidazole and nifurtimox. Gossypol was not able to eliminate *T. cruzi* epimastigotes completely in both strains at the studied doses. In these *in vitro* studies the MIGUZ strain was not affected by benznidazole and it was only partially susceptible to nifurtimox. At a concentration of 10 µM gossypol showed a better trypanocidal effect than benznidazole and nifurtimox in both strain. At the same concentration (10 µM), 4-isopropyl salicylaldehyde showed a trypanocidal effect two times higher than gossypol and the 4-isopropyl salicylic acid was the most active of all, showing a four times better trypanocidal effect than gossypol. In addition, all the epimatigotes were completely eliminated in both strains, although NINOA strain was eliminated more efficiently than the other strain. These experiments also indicated that 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde were able to penetrate the membrane of *T. cruzi* on account of the observed trypanocidal effect.

These compounds 4-isopropyl salicylic acid and 4-isopropyl salicylaldehyde reduced the mice parasitemia induced with the two strain of *T. cruzi* employed (*in vivo* trypanocidal activity). In these experiments 4-isopropyl salicylic acid showed better trypanocidal activity than benznidazole, nifurtimox and gossypol in mice infected with both strains. Although 4-isopropyl salicylaldehyde showed the same trypanocidal effects, in general, in mice infected with NINOA strain it showed a slightly lower effect than in the mice infected with MIGUZ strain. It was clearly observed that the *in vivo* trypanocidal activity of these compounds was less effective in mice infected with MIGUZ strain. this could be due to the partial resistance of the MIGUZ strain to these drugs, that it is not present in NINOA strain. Resistance to these drugs was observed in many *T. cruzi* strains.[17,18,23] This is the consequence of the existence of drug enzymatic detoxification systems in *T. cruzi*, similar to those that exist in mammalian liver, that easily eliminates or induce inactivation or loss of pharmacological activity in drugs. These enzymatic systems are present in susceptible and resistant *T. cruzi* strains and apparently the enzymatic levels of these systems are different.[16] Probably, the possible *in vitro* drug resistance observed in MIGUZ strain it is due to the fact that this strain metabolizes more efficiently these drugs than NINOA strain.

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