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# *In vitro* activity of 2-pyridinecarboxylic acid against trypanosomes of the subgenus *Schizotrypanum* isolated from the bat *Phyllostomus hastatus*

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**ABSTRACT.** The effect of 2-pyridinecarboxylic acid (picolinic acid) on trypanosomes of the subgenus *Schizotrypanum* isolated from the bat *Phyllostomus hastatus* was determined in this study. Picolinic acid, at 50 μg $mL^{-1}$, inhibited epimastigote growth by 99% after 12 days incubation. In addition, trypomastigote motility decreased by 50% after 6h and completely after 24h in the presence of 50 μg $mL^{-1}$ picolinic acid. The 50% cytotoxic concentration on HEp-2 cell line was 275 μg $mL^{-1}$ after 4 days incubation. Altogether, these results indicate higher toxicity against trypanosomes. The inhibitory effect of picolinic acid on epimastigote growth can be partially reversed by nicotinic acid and L-tryptophan, suggesting a competitive inhibition. Furthermore, two anti-*Trypanosoma* (*Schizotrypanum*) *cruzi* drugs were also evaluated with regard to bat trypanosome growth. Benznidazole, at 50 μg $mL^{-1}$, inhibited epimastigote growth by 90% after 12 days incubation. Nifurtimox, at the same concentration, caused 96% growth inhibition after four days incubation. Corroborating a previous study, bat trypanosomes are a good model for screening new trypanocidal compounds. Moreover, they can be used to study many biological processes common to human pathogenic trypanosomatids.

**Keywords:** bat trypanosome, *Schizotrypanum*, 2-pyridinecarboxylic acid, antimicrobial activity.

**RESUMO. Atividade *in vitro* do ácido 2-piridinocarboxílico em tripanossoma do subgênero *Schizotrypanum* isolado do morcego *Phyllostomus hastatus*.** O efeito do ácido 2-piridinocarboxílico (ácido picolínico) sobre um tripanossoma do subgênero *Schizotrypanum* isolado do morcego *Phyllostomus hastatus* foi determinado neste estudo. O ácido picolínico, na concentração de 50 μg $mL^{-1}$, inibiu 99% do crescimento de epimastigotas após 12 dias de incubação. Além disso, houve um decréscimo de 50 e 100% na mobilidade dos tripomastigotas após 6 e 24h, respectivamente, em presença de ácido picolínico na concentração de 50 μg $mL^{-1}$. A concentração citotóxica 50% para células HEp-2 foi de 275 μg $mL^{-1}$ após quatro dias de incubação. Esses resultados indicam maior toxicidade contra os tripanossomas. O efeito inibitório do ácido picolínico sobre o crescimento de epimastigotas pode ser parcialmente revertido por ácido nicotínico e L-triptofano, sugerindo inibição competitiva. Adicionalmente, o efeito de dois fármacos com atividade anti-*Trypanosoma* (*Schizotrypanum*) *cruzi* foi avaliado sobre o crescimento do tripanossoma de morcego. Benzonidazol, na concentração de 50 μg $mL^{-1}$, inibiu 90% do crescimento de epimastigotas após 12 dias de incubação. Nifurtimox, na mesma concentração, causou 96% de inibição do crescimento após quatro dias de incubação. Corroborando trabalhos anteriores, tripanossomas de morcegos são bons modelos para seleção inicial de novos compostos tripanocidas. Além disso, eles podem ser utilizados para estudar vários processos biológicos comuns aos tripanossomatídeos patogênicos ao homem.

**Palavras chave:** tripanossomas de morcegos, *Schyzotrypanum*, ácido 2-piridinocarboxílico, atividade antimicrobiana.

## Introduction

The subgenus *Schizotrypanum* includes several trypanosome species that are difficult to discriminate morphologically (HOARE, 1972). Whereas *Trypanosoma* (*Schizotrypanum*) *cruzi*, the causative agent of Chagas' disease, infects humans and a wide variety of mammalian hosts, all other *Schizotrypanum* species are restricted to bat species of the order Chiroptera (BARNABE et al., 2003).

Chagas' disease (CHAGAS, 1909) is an endemic disease affecting more than 15 million individuals in Latin America (GUHL; LAZDINS-HELDS, 2007)

This disease has a variable clinical manifestation ranging from symptomless infection to severe chronic disease with cardiovascular or gastrointestinal involvement (PRATA, 2001). There is no vaccine against *T. cruzi* infection, and the currently available drugs (nifurtimox and benznidazole) are active in acute or short-term chronic infections but have limited efficacy during the chronic phase of the disease (URBINA; DOCAMPO, 2003). Both drugs have severe side-effects which can lead to discontinuation of treatment, and strains that are naturally resistant to them have been reported (FILARDI; BRENER, 1987; LÉON-PÉREZ et al., 2007). Furthermore, *T. cruzi* develops benznidazole resistance during experimental infection in mice (SANTOS et al., 2008). Therefore, there is a need for the development of new and more efficacious anti-*T.* (*S.*) *cruzi* drugs.

Active antitrypanosomal compounds are usually detected by their suppressive effects on parasitemia and the decrease in mortality rates of experimentally infected animals (ROMANHA et al., 2010). Such an experimental system requires several laboratory procedures, which can thereby result in a high risk of accidental infection with *T.* (*S.*) *cruzi* and high costs. Therefore, *in vitro* tests using culture forms and growth inhibition criteria have lower costs, produce rapid results, and require small amounts of the compounds to be tested (DUARTE et al., 2002; IZUMI et al., 2008; ROMANHA et al., 2010; SCHLEMPER et al., 1977). In order to circumvent the risk of infection, trypanosomatids non-pathogenic to humans have been used as a model for the initial screening of antitrypanosomal drugs (BACCHI et al., 1974; BAKER; SELDEN, 1981; DUARTE et al., 2002; HOLETZ et al., 2003; MAGÁN et al., 2004; PEDROSO et al., 2006).

Bat trypanosomes of the subgenus *Schizotrypanum* have a cosmopolitan distribution and can be discriminated from *T.* (*S.*) *cruzi* by biological and genetic features (BAKER et al., 1978; BARNABE et al., 2003; HAMANAKA; PINTO, 1993; PETRY et al., 1986; PINTO et al., 1996; STEINDEL et al., 1998; TEIXEIRA et al., 1993). This protozoan can be easily cultivated in axenic medium, and in general, the growth characteristics are similar to those of *T.* (*S.*) *cruzi*. However, the bat trypanosome does not grow at 37°C, and it has not been detected in fresh blood samples or hemocultures of inoculated mice (BAKER et al., 1978; HAMANAKA; PINTO, 1993; PINTO et al., 1987). These features make bat trypanosomes suitable as a model for screening new trypanocidal compounds (DUARTE et al., 2002) and for studying biological events common to pathogenic trypanosomatids.

Picolinic acid is a naturally occurring end-product of L-tryptophan catabolism via the kynurenine pathway, and its production is catalyzed by picolinic carboxylase (MEHLER, 1956). Several functions have been attributed to picolinic acid, including cell cycle control (FERNANDEZ-POL et al., 1977), metal-chelation (EVANS; JOHNSON, 1980), antitumor activity in mice (LEUTHAUSER et al., 1982), and modulation of macrophage (BOSCO et al., 2003; MUCCI et al., 2003) and neutrophil (ABE et al., 2004) functions.

The effects of picolinic acid and derivatives on various microorganisms have been reported. Accordingly, *Escherichia coli* growth and *Bacillus subtilis* sporulation are transiently inhibited by picolinic acid because of its metal-chelating property (COLLINS et al., 1979; FORTNAGEL; FREESE, 1968). The growth of *Mycobacterium avium* complex organisms was also inhibited by picolinic acid (CAI et al., 2006). Antiviral activity against human immunodeficiency virus-1 and human herpes simplex virus-2 infected cells was demonstrated by Fernandez-Pol et al. (2001). In addition, picolinic acid is a potent costimulus in the induction of macrophage - or neutrophil-mediated microbicidal activity against *Candida albicans* (ABE et al., 2004; MUCCI et al., 2003) and *M. avium* (CAI et al., 2006; PAIS; APPELBERG, 2000).

In this study, the effect of 2-pyridinecarboxylic acid (picolinic acid), a catabolite of L-tryptophan, was tested against trypanosomes of the subgenus *Schizotrypanum* isolated from the bat *Phyllostomus hastatus.* The cytotoxicity of picolinic acid against HEp-2 (human larynx carcinoma squamous cell) cells was also evaluated.

## Material and methods

Reagents: The reagents used in this study were obtained from the following companies. Bayer (São Paulo State, Brazil): 3-methyl-4-(5'-nitrofurfurylidene-amino)-tetrahydro-4-2H-1,4-thiazine-1,1-dioxide (nifurtimox). Biobrás (Minas Gerais State, Brazil): brain heart infusion (BHI) medium. Microbiologica (Rio de Janeiro State, Brazil): fetal bovine serum. Roche Pharmaceuticals (Rio de Janeiro State, Brazil): N-benzyl-2-nitro-1H-imidazolacetamide (benznidazole). Sigma Chemical Co. (St Louis, USA): picolinic acid, L-tryptophan, nicotinic acid and nicotinamide.

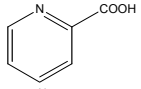
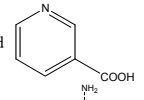
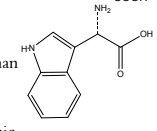
Preparation of solutions: Stock solutions (10 mg $mL^{-1}$) of L-tryptophan, nicotinic acid, picolinic acid and nicotinamide were prepared in water, and benznidazole and nifurtimox were prepared in 10% DMSO (v $v^{-1}$). These solutions were sterilized by filtration (0.22 µm, Millipore, São Paulo State, Brazil) and added aseptically only once to growth medium at determined concentrations.

Microorganism: Stock M5 of *Schizotrypanum* was isolated from the bat *Phyllostomus hastatus* which was captured in Lapa Vermelha cavern in Pedro Leopoldo, Minas Gerais State, Brazil (PINTO et al., 1987). This isolate was cultured in blood agar medium, and M5-2 strain was obtained and used in all experiments of this study. Epimastigote forms of the flagellate were maintained at 28ºC in BHI medium supplemented with 10% (v $v^{-1}$) heat-inactivated fetal bovine serum and 0.2% (v $v^{-1}$) rabbit hemoglobin, pH 7.3 (growth medium).

Antitrypanosomal activity: Epimastigotes of M5-2 strain in logarithmic growth phase (5 x $10^5$ cells) were added to 18 x 180 mm screw-capped tubes containing 2 mL of growth medium with 10 or 50 µg $mL^{-1}$ picolinic acid added. The cultures were incubated at 28ºC, and cell growth was estimated by direct counting in a hemocytometer (Improved Double Neubauer) at 24h intervals for 12 days. Benznidazole and nifurtimox were used as reference drugs. Trypomastigote forms were obtained by spontaneous differentiation at 28ºC of M5-2 epimastigotes in growth medium (15 to 20 days). These parasites were then harvested by centrifugation at 1500 g for 10 min., washed in sterile 10 mM phosphate buffer, pH 7.2, and cells counted. Parasites (5 x $10^5$ cells) were incubated with 10 or 50 µg $mL^{-1}$ picolinic acid in the same buffer at 28ºC, and cell motility was observed with a light microscope after 4, 6, 12 and 24h. The results were expressed as described in the legend of Table 1. All experiments were performed in duplicate on three different occasions. Growth medium (or buffer) alone or containing a determined DMSO concentration was used as the negative control.

Cytotoxicity assay: HEp-2 cells (human larynx carcinoma, ATCC, CCL-23) were cultured in Dulbecco´s modified Eagle´s medium [DMEM (Invitrogen-Gibco, USA)] supplemented with 10% (v $v^{-1}$) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU $mL^{-1}$ penicillin, 100 µg $mL^{-1}$ streptomycin and 2.5 µg $mL^{-1}$ amphoterecin B, in 5% $CO_2$ at 37ºC. The cells were added to 96 well culture plate at a density of 1 x $10^5$ cells/well and incubated for 24h. At confluence, non-adherent cells were removed by washing with sterile PBS. The medium containing different concentrations o picolinic acid (1200 – 1.12 µg $mL^{-1}$) were added to each well containing the cells, and the plates were incubated for 96h. Cell viability was determined by the MTT [dimethylthiazol diphenyl tetrazolium bromide (Sigma Chemical Co., USA)] method according to the manufacture´s recommendation. The concentration of the compounds needed to inhibit the viable cells up to 50% by regression analysis corresponds to the 50% cytotoxic concentration ($CC_{50}$) after 4 days incubation.

**Table 1.** Effect of picolinic acid on motility of trypomastigote forms from bat trypanosomes.

| Systems | Chemical structure | Concentration (µg $mL^{-1}$) | Time (h) 0 | 6 | 12 | 24 |
|---|---|---|---|---|---|---|
| None | | | +++ | +++ | +++ | +++ |
| Picolinic acid (PA) |  | 10 | +++ | +++ | +++ | +++ |
| | | 50 | +++ | ++ | + | - |
| Nicotinic acid |  | 100 | +++ | +++ | +++ | +++ |
| L-Tryptophan |  | 100 | +++ | +++ | +++ | +++ |
| PA+nicotinic | | 50 + 100 | +++ | +++ | ++ | + |
| PA+L-tryptophan | | 50 + 100 | +++ | +++ | ++ | ++ |

The compounds were tested as described in Material and Methods. The results are th average of three determinations and expressed as number of motile trypomastigotes +++, the same number of motile trypomastigotes as the control; ++, less than 50% o the control; +, less than 10% of the control; -, no mobile trypomastigotes were seen.

Effect of L-tryptophan, nicotinic acid and nicotinamide on picolinic acid inhibitory activity. The experiments using epimastigote and trypomastigote forms were performed as above except that L-tryptophan, nicotinic acid and nicotinamide were added (100 µg $mL^{-1}$ final concentration) aseptically to the growth medium or phosphate buffer containing 50 µg $mL^{-1}$ picolinic acid. Controls in the absence of picolinic acid were performed. The results were evaluated by the Tukey-Kramer test, using the software Statistics for Windows, version 6.0 (Statsoft, Inc., Oklahoma city, Okla., USA). A p value less than 0.05 was considered significant.

## Results and discussion

Bat trypanosomes can be separated into three distinct groups using genetic markers - two from Europe (*Trypanosoma dionisii* and *Trypanosoma vespertilionis*) and one from the Americas (*Trypanosoma marinkellei*). The latter is more related to *T.* (*S.*) *cruzi* than the European isolates (BAKER et al., 1978

BARNABE et al., 2003), but as mentioned, bat trypanosomes do not grow under conditions that exist in humans (BAKER et al., 1978; HAMANAKA; PINTO, 1993; PINTO et al., 1987). Therefore, bat trypanosomes can be used for the initial screening of new antitrypanosomal drugs (DUARTE et al., 2002). In support of this notion, we determined the susceptibility of bat trypanosomes to drugs used in the treatment of Chagas' disease. Benznidazole and nifurtimox are active against epimastigote forms of bat trypanosomes, producing a high percentage of growth inhibition at both drug concentrations tested (10 and 50 μg $mL^{-1}$). While benznidazole causes growth inhibition greater than 90% after 12 days incubation, nifurtimox inhibited growth by 96% after two and four days incubation in the presence of 10 and 50 μg $mL^{-1}$, respectively.

In this study, we also examined the effect of picolinic acid on the growth of epimastigote forms and the motility of trypomastigote forms of M5-2 strain of *Schizotrypanum* trypanosomes isolated from bat *Phyllostomus hastatus*. An inhibitory effect on cell growth was observed when epimastigotes of bat trypanosomes were treated with 50 μg $mL^{-1}$ picolinic acid, and approximately 50% inhibition was detected after 4 days incubation. After 12 days incubation, this compound inhibited epimastigote's growth by 99% (Figure 1).

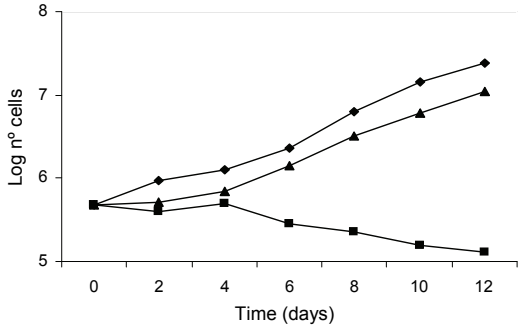



**Figure 1.** Effect of picolinic acid on growth of bat trypanosomes of the subgenus *Schizotrypanum*. Epimastigote forms were cultivated at 28ºC in growth medium supplemented with 10 and 50 μg $mL^{-1}$ picolinic acid, and cell growth was estimated by direct counting in a hemocytometer at 24h intervals for 12 days. Growth medium alone was used as control. The results are the average of three experiments in duplicate and expressed as log of cell numbers. (◆) Control cells; (▲) 10 μg $mL^{-1}$; and (■) 50 μg $mL^{-1}$.

The number of motile trypomastigotes decreased (50%) after 6h in the presence of 50 μg $mL^{-1}$ picolinic acid, and after 24h no motile trypomastigotes were observed. This effect was delayed when 100 μg $mL^{-1}$ of nicotinic acid or L-tryptophan were added with 50 μg $mL^{-1}$ picolinic acid. No significant effect on the trypomastigotes' motility was observed in the presence of only 10 μg $mL^{-1}$ picolinic acid, and 100 μg $mL^{-1}$ of nicotinic acid or L-tryptophan (Table 1).

The 50% cytotoxic concentration of picolinic acid after 4 days incubation ($CC_{50/4d}$) of HEp-2 cells was 275 μg $mL^{-1}$, indicating higher selective toxicity of the compound against trypanosomes compared to mammalian cells.

The mechanisms of action of picolinic acid regarding the inhibitory effects on M5-2 trypanosomes are unclear. 3-Mercaptopicolinic acid, a structural analogue of picolinic acid, has been reported to be an inhibitor of phosphoenolpyruvate carboxykinase from hepatic cells of rats and guinea pigs (JOMAIN-BAUM et al., 1976) and birds (MAKINEN; NOWAK, 1983). This enzyme catalyzes the reversible conversion of oxalacetate to phosphoenolpyruvate, a central reaction of gluconeogenesis, and has a strict requirement for divalent metal ions for activity (UTTER; KOLENBRANDER, 1972). 3-Mercaptopicolinic acid also inhibits phosphoenolpyruvate carboxykinase from *T.* (*S.*) *cruzi* (URBINA et al., 1990). However, in contrast to its role in other organisms, this enzyme has been shown to be responsible for the incomplete degradation of carbohydrates, and it is an important enzyme in amino acid catabolism and energy production in *T.* (*S.*) *cruzi* (URBINA, 1994).

On the other hand, picolinic acid is a structural analogue of nicotinic acid (3-pyridinecarboxylic acid) and nicotinamide which are supplied in chemically defined medium for trypanosomatid growth (MELO et al., 1985; ROITMAN et al., 1972). Nicotinic acid and nicotinamide are precursors of an essential cellular cofactor, nicotinamide adenine dinucleotide (NAD), and in their absence, L-tryptophan can be a source for de novo NAD biosynthesis (MOFFETT; NAMBOODIRI, 2003; NISHIZUKA; HAYAISHI, 1963). These cofactors can participate in many critical cellular reactions, including energy production (NELSON; COX, 2000) and DNA repair (WACHSMAN, 1996).

The effects of picolinic acid are partially reversed by nicotinic acid (Figure 2A, $p < 0.001$) and L-tryptophan (Figure 2B, $p < 0.01$). Therefore, it is possible that picolinic acid could compete with nicotinic acid and alter its cell functions. Nishizuka and Hayaishi (1963) showed that NAD biosynthesis from L-tryptophan in mammalian hepatic and kidney cells appears to be inversely related to picolinic carboxylase activity. Interestingly, our study showed that nicotinamide did not prevent the growth

inhibitory effect of picolinic acid on trypanosomes (data not shown). Bat trypanosomes probably synthesize NAD only from nicotinic acid. Further studies are needed to elucidate the mechanism of the antitrypanosomal activity of picolinic acid.

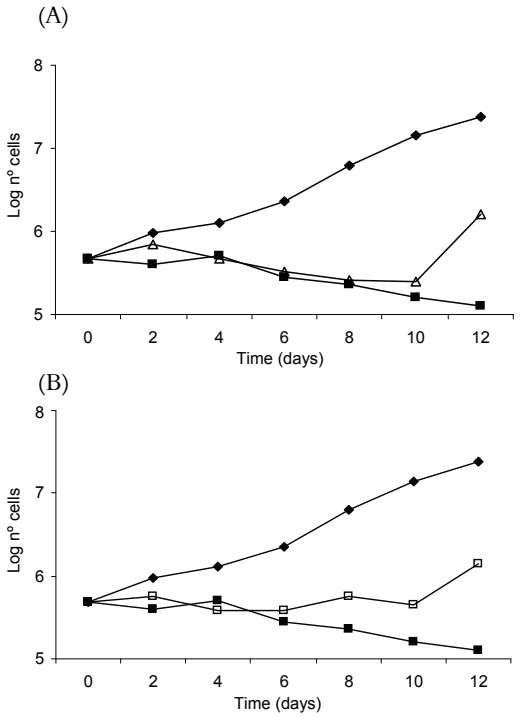



**Figure 2.** Effect of nicotinic acid (A) and tryptophan (B) on growth inhibition of picolinic acid. Epimastigote forms were cultivated at 28°C in growth medium supplemented with 50 μg $mL^{-1}$ picolinic and 100 μg $mL^{-1}$ nicotinic acid, and cell growth was estimated by direct counting in a hemocytometer at 24h intervals for 12 days. Growth medium alone was used as control. The results are the average of three experiments in duplicate and expressed as log of cell numbers. (◆) Control cells; (■) Picolinic acid; (Δ) Picolinic acid + nicotinic acid ($p < 0.001$); and (□) Picolinic acid + L-tryptophan ($p < 0.01$).

## Conclusion

As mentioned, various studies utilize non-pathogenic trypanosomatids as useful experimental models which can help in the understanding of the biology, physiology and host-parasite relationship of species pathogenic to humans (LIU et al., 2005; MITTRA; RAY, 2004). Our results showed that this flagellate is sensitive to drugs that are active against *T.* (*S.*) *cruzi*, demonstrating their usefulness as a reliable model for the initial selection of substances with potential inhibitory activity on the growth of this pathogenic microorganism, as described by others (DUARTE et al., 2002).

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