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FEMS Microbiology Letters 226 (2003) 379–384

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Effects of metronidazole and tinidazole on NTPDase1 and ecto-5′-nucleotidase from intact cells of *Trichomonas vaginalis*

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Received 17 July 2003; accepted 11 August 2003

First published online 2 September 2003

Abstract

Here we report the effects of metronidazole and tinidazole on NTPDase1 and ecto-5′-nucleotidase from intact cells of *Trichomonas vaginalis*. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) hydrolysis was 5- to 7-fold higher for the fresh clinical strain, when compared with the ATCC (American Type Culture Collection) strain. ATP hydrolysis was activated in the presence of metronidazole in the ATCC strain, whilst it was inhibited 33% by 50 μM tinidazole in a fresh clinical isolate. The treatment of cells in the presence of metronidazole for 2 h inhibited ATP and ADP hydrolysis, whilst treatment with tinidazole inhibited ATP and ADP hydrolysis only in the fresh clinical isolate. The drugs did not change the ecto-5′-nucleotidase activity for both strains. Our results suggest that the modulation of extracellular ATP and ADP levels during treatment with these drugs could be a parasitic defence strategy as a survival mechanism in an adverse environment.

Keywords: NTPDase1; Ecto-5′-nucleotidase; Metronidazole; Tinidazole; *Trichomonas vaginalis*

1. Introduction

Trichomonas vaginalis is emerging as a major pathogen in men and women and is associated with serious health consequences [1]. Trichomoniasis is now the most common non-viral sexually transmitted disease in the world [2]. Recent publications show that *T. vaginalis* promotes the transmission of human immunodeficiency virus (HIV) [3,4], causes low-weight and premature birth [5], predisposes women to atypical pelvic inflammatory disease [6], cervical cancer [7–9] and infertility [10]. *T. vaginalis* possesses multiple mechanisms for colonisation of the vaginal tract and achieves successful host parasitism through various mechanisms including: (1) acquisition of nutrients through specific receptors; (2) contact with the vaginal epithelium where four iron-regulated surface proteins mediate cytoadherence to the vaginal epithelial cells; (3) evasion of immune responses; (4) alternating surface expression of at least two antigen repertoires; and (5) alternate and coordinate expression of virulence genes in response to host environmental factors [11]. Indeed, the proteins and glycoproteins on the cell surface of the trichomonad play a major role in the mechanisms of its pathogenesis, adhesion, host–parasite interaction and nutrient acquisition.

Extracellular adenosine triphosphate (ATP) may act as a signalling compound in cytolytic mechanisms [12,13] and it is hydrolysed to adenosine by ectonucleotidases. Several studies have reported the presence of ectonucleotidases on the surface of parasites. Recently, we characterised NTPDase1 [14] and 5′-nucleotidase activities in *T. vaginalis* (Tasca et al., submitted). The NTPDase1 (apyrase,

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0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00637-2

ecto-ATP diphosphohydrolase, CD39, EC 3.6.1.5) hydrolyses nucleoside di- and triphosphates, producing nucleoside monophosphates and inorganic phosphate. In addition, the ecto-5′-nucleotidase (CD73, EC 3.1.3.5), hydrolyzes nucleoside monophosphates, forming the nucleoside adenosine [15,16]. The presence of enzymes performing ATP, adenosine diphosphate (ADP) [14] and adenosine monophosphate (AMP) hydrolysis in trichomonads may be important for: (a) modulation of nucleotide concentration in the extracellular space, (b) protection from the cytolytic effects of extracellular ATP, (c) involvement in signal transduction, and (d) involvement in cellular adhesion. In addition, *T. vaginalis* lacks the ability to synthesise purines and pyrimidines de novo and their growth and survival are dependent upon salvage pathways to generate nucleotides [17,18].

Metronidazole was the first nitroimidazole to show useful clinical activity against many microaerophilic microorganisms and, since 1960, it is still the drug of choice for trichomoniasis [19]. Other nitroimidazoles, although unavailable in North America, are also approved for clinical use in other parts of the world. These include tinidazole, ornidazole, secnidazole, flunidazole, nimorazole and carnidazole [20]. The 5-nitroimidazoles are administered as inactive prodrugs and are converted to cytotoxic forms by susceptible organisms; the drugs are ineffective in aerobic microorganisms or mammalian cells, and this is the basis of its selective toxicity [21]. These compounds require reduction of the nitro group in order to kill susceptible cells, resulting in production of toxic intermediates. The knowledge of mechanisms by which short-lived nitroimidazole radicals kill susceptible organisms is rather incomplete [22]. Some experimental evidence, however, indicates that one of the drug's targets is DNA, where a short-lived reduction product, most probably the protonated one-electron nitro radical anion, oxidises DNA causing strand breaks and subsequent cell death [19]. Damage to other vital systems of the cell may, however, be anticipated, but the direct evidence is missing [22]. In this study, we investigated the effect of metronidazole and tinidazole on the NTPDase1 and the ecto-5′-nucleotidase activities in intact cells from the ATCC strain and from a fresh clinical isolate of *T. vaginalis*.

2. Materials and methods

2.1. Parasites and culture conditions

The 30236 strain from the American Type Culture Collection (ATCC) and the POA-3 strain, a fresh clinical isolate of *T. vaginalis*, were used in this study. The strains were cultivated axenically in trypticase-yeast extract-maltose (TYM) medium [23] without agar (pH 6.0) supplemented with 10% (v/v) heat-inactivated bovine serum, penicillin (1000 IU ml^{-1}) and streptomycin sulphate (1 mg ml^{-1}) in aerobiosis at 37°C (±0.5°C). Isolates were subcultured every 48 h in TYM medium. Trichomonads in the logarithmic phase of growth and within 48 h of subculture (exhibiting more than 95% mobility and normal morphology) were harvested and washed with sterile saline solution (0.85% NaCl) (750×*g* for 5 min) three times. Parasites were resuspended in saline solution and counted with a haemocytometer and adjusted to a concentration of 1.5×10^6 cells ml^{-1}, corresponding to a concentration of 0.3–0.7 mg ml^{-1} of protein.

2.2. Cell viability

All experiments were performed using intact cells and cellular viability was assessed before and after incubations, by mobility and trypan blue dye exclusion [24]. A 20 μl aliquot of cells was collected from the enzymatic assays and mixed with 20 μl 0.4% trypan blue dye. The cells were counted in a haemocytometer. Trypan blue-stained cells were considered as inviable regardless of the degree of blue [25]. The viability was not affected by incubation conditions.

2.3. In vitro susceptibility assay

Previous experiments were performed in order to evaluate the in vitro susceptibility of trichomonads to the nitroimidazoles as described by Tachezy et al. [26]. Trichomonads from the log phase of growth were exposed to 2-fold serial dilutions of metronidazole and tinidazole in the presence of air or under anaerobiosis for 48 h at 37°C. Standard microtitre plates with eight rows of 12 round-bottom wells were used in the test. The TYM medium was added in 50 μl aliquots to each well. Stock solutions of metronidazole and tinidazole were added in 50 μl aliquots to the wells of the first row. Starting from the wells of the first column 2-fold serial dilutions were made. Finally, 5×10^4 cells ml^{-1} from 30236 and POA-3 strains were placed into each well. The plates were incubated aerobically in a humid chamber or in an anaerobic jar (Probac) containing hydrogen-generating envelopes (Anaerobac). Controls with trichomonads, but without addition of drugs were included. The susceptibility test was performed in triplicate for each drug, and with two different cell suspensions, for both strains. After 48 h, the contents of the wells were homogenised and an aliquot was examined under a light microscope at 400× for motile trichomonads.

2.4. NTPDase1 and ecto-5′-nucleotidase activities

Intact cells of *T. vaginalis*, the ATCC 30236 and the fresh clinical isolate, POA-3, were added to the reaction mixture containing 50 mM Tris buffer (pH 7.2), 5 mM $CaCl_2$, for measuring NTPDase1 activity. For ecto-5′-nucleotidase activity, the cells of both strains were added to

the reaction mixture containing 50 mM Tris buffer (pH 7.5) and 3 mM $MgCl_2$. Metronidazole or tinidazole (10, 25 and 50 μM) was added in both reaction media. The samples were preincubated for 5 min at 37°C in 200 μl of the reaction mixture. The reaction for NTPDase1 activity was initiated by the addition of ATP or ADP to a final concentration of 1 mM, while the reaction for ecto-5′-nucleotidase was initiated by the addition of AMP to a final concentration of 3 mM. The reaction of hydrolysis of ATP and ADP was stopped after 40 min and the reaction of hydrolysis of AMP was stopped after 60 min by adding 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) [27]. Incubation times and cellular density were chosen in order to ensure the linearity of the reactions. Controls with the addition of the intact cells after mixing trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol Pi released per min per 1.5×10^6 cells. All samples were run in triplicate, with similar results achieved in at least three different cell suspensions.

2.5. Treatment of cells

In order to investigate the effect of the drugs after a treatment, the cells were kept in presence of metronidazole or tinidazole (50 μM) for 2 h, before washing with sterile saline solution (0.85% NaCl) ($750 \times g$ for 5 min) three times. The subsequent steps for incubation to measure the nucleotide hydrolysis were the same as described above.

2.6. Protein determination

Protein was measured by the Coomassie blue method [28], using bovine serum albumin as standard.

2.7. Statistical analysis

Statistical analysis was conducted by one-way ANOVA (analysis of variance) and Student's *t*-test, considering a level of significance of 5%.

2.8. Chemicals

Nucleotides, Tris buffer, trypan blue dye and metronidazole were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and tinidazole was a gift from Medley, Brazil. Fresh stock solutions for assays of both drugs were prepared before each experiment. Tinidazole (5 mM) was dissolved in water and metronidazole (5 mM) was dissolved in water by autoclaving (1 atm, 121°C, 15 min) [26] and after these solutions of both drugs were diluted in water to obtain the final concentrations (10, 25, and 50 μM). All other reagents were of the highest analytical grade available.

3. Results and discussion

In the present study we investigated the effects of metronidazole and tinidazole on the NTPDase1 and the ecto-5′-nucleotidase in intact cells of *T. vaginalis*. Cellular integrity and viability were assessed before and after the reactions, by the mobility of trophozoites and trypan blue dye exclusion. The integrity of the cells was not affected by any of the conditions used in the assays.

The in vitro susceptibility to metronidazole and tinidazole was tested for 30236 and POA-3 strains. The strains were susceptible to the drugs in all tested concentrations (data not shown).

The enzymatic activities were measured in one ATCC strain and one fresh clinical isolate of *T. vaginalis*. AMP hydrolysis demonstrated no difference between the investigated strains. Interestingly, ATP and ADP hydrolysis were higher (7- and 5-fold, respectively) in the fresh clinical isolate when compared with the hydrolysis of the ATCC strain, 30236. The NTPDase1 is probably involved in cell pathogenesis, considering the lower time of cultivation of the fresh isolate that maintains the main biological

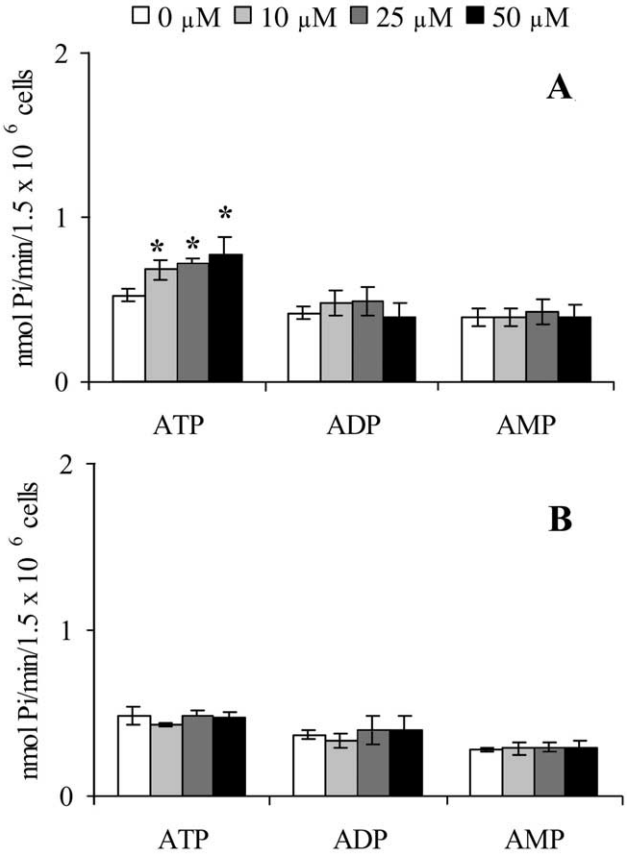

Fig. 1. Effect of metronidazole (A) and tinidazole (B) on ATP, ADP and AMP hydrolysis in the 30236 strain. Bars represent the mean ± S.D. of at least three experiments using different cell suspensions, each in triplicate. Results were analysed statistically by one-way ANOVA. *Significant difference from controls by one-way ANOVA ($P < 0.05$).

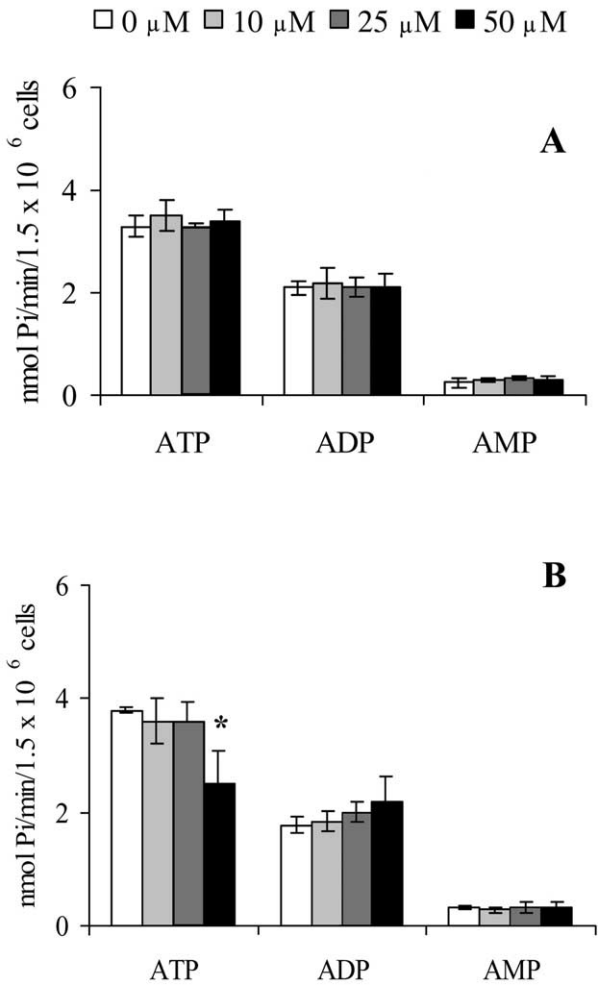

Fig. 2. Effect of metronidazole (A) and tinidazole (B) on ATP, ADP and AMP hydrolysis in the POA-3 strain. Bars represent the mean ± S.D. of at least three experiments using different cell suspensions, each in triplicate. Results were analysed statistically by one-way ANOVA. *Significant difference from controls by one-way ANOVA ($P < 0.05$).

features of the parasite as in the host site conditions. On the other hand, ATCC cells may present changes in their biochemical metabolism including pathogenesis and immune evasion, due to their maintenance in axenic culture for several years.

Here, in vitro studies were conducted to provide more information regarding the direct interaction of nitroimidazole compounds and the ecto-enzymes involved in nucleotide hydrolysis in *T. vaginalis*. Fig. 1 shows the NTPDase1 (ATP and ADP as substrates) and the ecto-5′-nucleotidase (AMP as substrate) activities measured in the presence of metronidazole (Fig. 1A) and tinidazole (Fig. 1B), for the 30236 strain. ATP hydrolysis was activated by 31, 38, and 47% in the presence of all tested concentrations of 10, 25, 50 μM metronidazole, respectively (Fig. 1A). On the other hand, ADP and AMP hydrolysis did not present significantly changes in the presence of metronidazole (Fig. 1A). Tinidazole did not significantly change the ATP, ADP and AMP hydrolysis at the concentrations tested when compared to the control enzyme activity (no drug added), with the 30236 strain (Fig. 1B). Likewise, the enzymatic activities were tested in the presence of the nitroimidazoles for the POA-3 strain, as shown in Fig. 2. Metronidazole did not significantly change the ATP, ADP and AMP hydrolysis (Fig. 2A) and tinidazole did not change the ADP and AMP hydrolysis (Fig. 2B), with the POA-3 strain. In contrast, ATP hydrolysis of the POA-3 strain was affected by tinidazole 50 μM, revealing an inhibition of 33% (Fig. 2B). These effects of nitroimidazole derivatives in the ATP hydrolysis could be the result of pharmacological interaction between the enzyme and drugs tested. Here, in order to explain the activation of ATP hydrolysis by metronidazole and the inhibition of ATP hydrolysis by tinidazole, two possibilities could be suggested: (a) these effects involved the participation of an ecto-ATPase, coexpressed with the NTPDase1 in *T. vaginalis*, as observed in rat brain [29], or (b) these effects occurred only in the ATPase activity of the NTPDase1.

The treatment of the 30236 strain in the presence of metronidazole for 2 h presented statistically significant in-

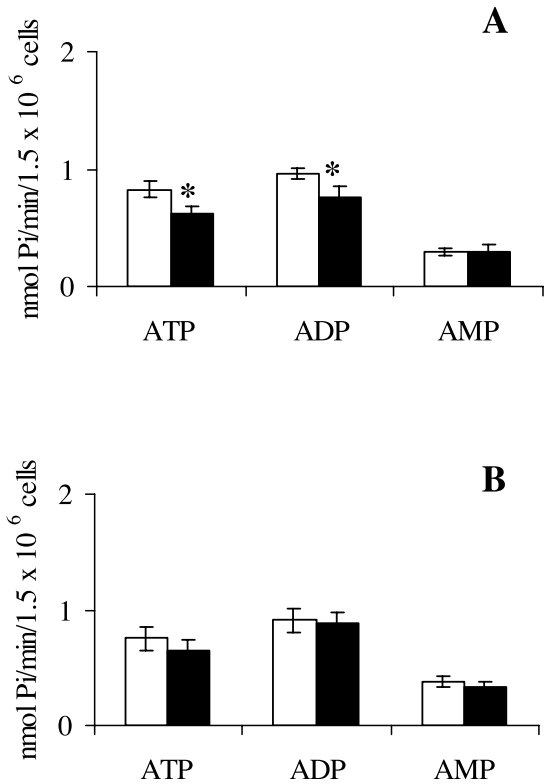

Fig. 3. Effect of metronidazole (A) and tinidazole (B) on ATP, ADP and AMP hydrolysis in the 30236 strain after 2 h of cell treatment. White bars represent controls (without drugs) and black bars represent 50 μM metronidazole or 50 μM tinidazole. Bars represent the mean ± S.D. of at least three experiments using different cell suspensions, each in triplicate. Results were analysed statistically by Student's *t*-test. *Significant difference from controls by Student's *t*-test ($P < 0.05$).

hibitions of 25 and 21% for ATP and ADP hydrolysis, respectively (Fig. 3A). In a similar condition, tinidazole did not significantly change the NTPDase1 and the ecto-5′-nucleotidase activities at the concentrations studied when compared to the control enzyme activity (no drug added), in the 30236 strain (Fig. 3B).

Fig. 4 shows the treatment of the POA-3 strain in the presence of metronidazole and tinidazole for 2 h. Metronidazole significantly changed the ATP and ADP hydrolysis, demonstrating inhibitions of 22 and 25%, respectively (Fig. 4A). The treatment with tinidazole presented statistically significant inhibitions of 19% for ATP and 17% for ADP (Fig. 4B).

Metronidazole and tinidazole did not significantly change the ecto-5′-nucleotidase activity at the concentrations studied when compared to the control enzyme activity (no drug added). This finding was similar for both strains, 30236 and POA-3.

When the cells were treated with metronidazole for 2 h, inhibition of ATP and ADP hydrolysis in both strains was observed, whilst tinidazole inhibition was seen only in the POA-3 strain. Previous studies have shown changes in activities of hydrogenosomal and cytosolic enzymes of *T. vaginalis* related to development of resistance to metronidazole [22]. Hydrogenosomal enzymes, pyruvate:ferredoxin oxidoreductase, hydrogenase, malic enzyme (hydrogenosomal L-malate:nicotinamide adenine dinucleotide (phosphate) (NAD(P)) oxidoreductase), and NAD:ferredoxin oxidoreductase are downregulated in metronidazole-resistant cells [30,31]. Conversely, cytosolic enzymes such as lactate dehydrogenase and pyruvate kinase are upregulated in trichomonads resistant to metronidazole [32,33]. In this study, we investigated the behaviour of enzymes involved in the breakdown of extracellular nucleotides in *T. vaginalis* strains sensitive to nitroimidazoles, after a pharmacological interaction with metronidazole and tinidazole. The inhibition in NTPDase1 activity after the treatment was probably due to an attempt by the parasite to modulate the nucleotide concentration in the extracellular space. This phenomenon could be involved in signal transduction, since the enzyme activities measured are involved in the cleavage of ATP to adenosine. As well as its energetic function, extracellular ATP has several effects on many biological processes, these include smooth muscle contraction, neurotransmission, immune response, inflammation, platelet aggregation, and pain [34–37]. Adenosine induces vasodilation, a decrease in glomerular filtration rate, inhibition of neurotransmitter release, inhibition of the immune and inflammatory response or lipolysis. In more primitive organisms, the bacterial 5′-nucleotidase has an ecological function in nutrient recycling in aqueous habitats [38].

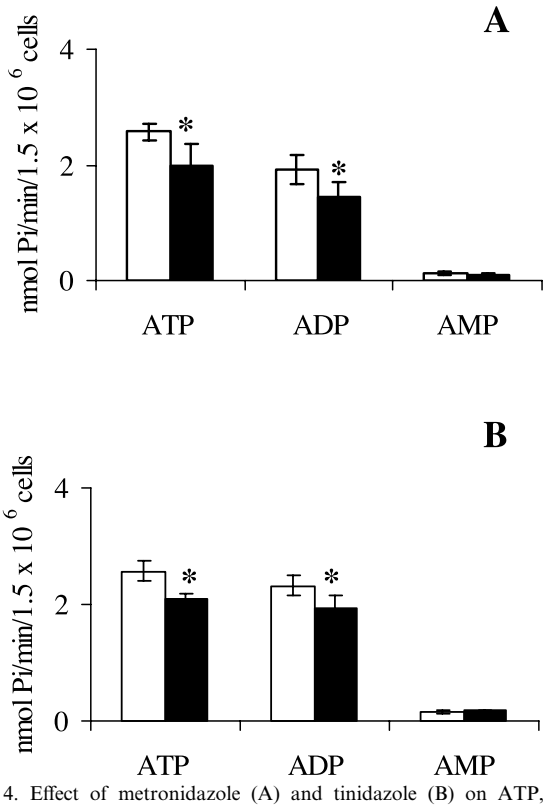

Fig. 4. Effect of metronidazole (A) and tinidazole (B) on ATP, ADP and AMP hydrolysis in the POA-3 strain after 2 h of cell treatment. White bars represent controls (without drugs) and black bars represent 50 μM metronidazole or 50 μM tinidazole. Bars represent the mean ± S.D. of at least three experiments using different cell suspensions, each in triplicate. Results were analysed statistically by Student's *t*-test. *Significant difference from controls by Student's *t*-test ($P < 0.05$).

Interestingly, tinidazole did not cause any alteration in the NTPDase1 activity in 30236 cells, only in the POA-3 strain. The difference between both strains reinforces the important use of fresh clinical isolates in experimental investigations, in addition to standard strains.

To our knowledge, this is the first report of the effects of 5-nitroimidazoles on the NTPDase1 and ecto-5′-nucleotidase activities in parasites. Our results suggest that the modulation of extracellular ATP and ADP levels during treatment with these drugs could be a parasite defence strategy as a survival mechanism in an adverse environment. More detailed studies will be necessary to clarify the importance and the significance of the results here presented.

Acknowledgements

Grateful thanks to Dr. Sérgio De Meda Lamb, Chairman of the School of Pharmacy (PUCRS) for his support and to Rafael V. Michel for technical assistance. This work was partially supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and the Third World Academy of Science (TWAS). T.T. is recipient of a CNPq fellowship.

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