Trypanosoma cruzi: In vitro effect of aspirin with nifurtimox and benznidazole

Rodrigo López-Muñoz a, Mario Faúndez d, Sebastián Klein d, Sebastián Escañilla d, Gloria Torres a, Dasfne Lee-Liu a, Jorge Ferreira a, Ulrike Kemmerling b, Myriam Orellana a, Antonio Morello a, Arturo Ferreira c, Juan D. Maya a,∗

a Program of Molecular and Clinical Pharmacology, ICBM, School of Medicine, University of Chile, Chile
b Program of Immunology, ICBM, School of Medicine, University of Chile/School of Health Sciences, Talca University, Chile
c Program of Anatomy and Developmental Biology, ICBM, School of Medicine, University of Chile

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A B S T R A C T

Nifurtimox and benznidazole are the only active drugs against Trypanosoma cruzi; however, they have limited efficacy and severe side effects. During primoinfection, T. cruzi infected macrophages mount an antiparasitic response, which the parasite evades through an increase of tumor growth factor β and PGE2 activation as well as decreased iNOS activity. Thus, prostaglandin synthesis inhibition with aspirin might increase macrophage antiparasitic activity and increase nifurtimox and benznidazole effect.

Aspirin alone demonstrated a low effect upon macrophage antiparasitic activity. However, isobolographic analysis of the combined effects of aspirin, nifurtimox and benznidazole indicated a synergistic effect on T. cruzi infection of RAW cells, with combinatory indexes of 0.71 and 0.61, respectively.

The observed effect of aspirin upon T. cruzi infection was not related with the PGE2 synthesis inhibition. Nevertheless, NO levels were restored by aspirin in T. cruzi-infected RAW cells, contributing to macrophage antiparasitic activity improvement.

Thus, the synergy of aspirin with nifurtimox and benznidazole is due to the capability of aspirin to increase antiparasitic activity of macrophages.

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1. Introduction

In Latin America, Chagas’ disease, caused by the protozoan Trypanosoma cruzi, represents a health threat for an estimated 10–20 million people and is the second highest burden of disease among Tropical Diseases in the Americas (Reithinger et al., 2009).

Current Chagas’ disease treatment is limited to nifurtimox and benznidazole. However, these drugs have limited efficacy and frequent and significant side effects. Hundreds of natural and synthetic compounds have been tested against T. cruzi. However, very few are devoid of host’s cytotoxic activity and are more effective than nifurtimox and benznidazole, especially against the intracellular amastigotes (Maya et al., 2007).

During primoinfection, T. cruzi trypanomastigotes invade macrophages, and transform in the intracellular amastigote forms. In this setting, macrophages mount an antiparasitic response through an inflammatory activity including increasing nitric oxide production and oxidative stress through peroxynitrite formation (Borges et al., 1998; Peluffo et al., 2004). Simultaneously, T. cruzi induces an anti-inflammatory response through activation of cellular mediators such as tumor growth factor β (TGF-β) (Li et al., 2006) and prostaglandins, specifically, PGE2 (Abdalla et al., 2008). TGF-β and PGE2 decrease iNOS activity and, consequently, increase polyamine production, by increasing L-arginine availability (Freire-de-Lima et al., 2006; Peluffo et al., 2004). Polyamines are essential for T. cruzi amastigote proliferation and trypanothione (T(SH)2) synthesis. T(SH)2 is the most important oxidative aggression scavenger in trypanosomatids (Maya et al., 2007). Thus, the parasite evades the innate immune response attenuating the antiparasitic response of macrophages by decreasing nitric oxide production, improving polyamine availability and providing a favorable intracellular redox environment.

Freire-de-Lima et al. (2000) showed that prostaglandin synthesis inhibition in T. cruzi infected macrophages, which have ingested apoptotic bodies, produced a significant decrease in infection. Further evidence indicates that prostaglandin synthesis inhibition could be important in acute infection therapy (Freire-de-Lima et al., 2000; Hideko Tatakihara et al., 2008; Michelin et al., 2005; Pinge-Filho et al., 1999).

In this work, we show that a COX inhibitor like aspirin (ASA) increases the trypanocidal activity of nifurtimox and benznidazole in
an *in vitro* model of *T. cruzi* infection. Thus, the experimental basis for a potentially new therapeutic strategy for the control of this disease is established.

### 2. Materials and methods

#### 2.1. Cell culture and *in vitro* infection with *T. cruzi*

RAW (1 × 10^5^) 264.7 cells/cm² were cultured in 0.22% sodium bicarbonate, 5% fetal bovine serum supplemented RPMI 1640 medium in humidified air with 5% CO₂ at 37 °C. RAW 264.7 cells were infected with Dm28c trypomastigotes, at a 1:3 (cell:parasite) ratio. *T. cruzi* trypomastigotes were initially obtained from primary cultures of peritoneal macrophage from chagasic mice.

#### 2.2. Cell viability assay

The effect of drug treatments on RAW 264.7 cells was evaluated through the MTT assay as viability test (Mosmann, 1983). Briefly, 10 µl of 5 mg/ml tetrazolium dye (MTT; 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) plus 0.22 mg/ml Phenazine metosulphate (electron carrier), were added to each well containing RAW 264.7 cell culture in 100 µl RPMI 1640 without phenol red. Drugs, dissolved in DMSO, were added to culture media at the concentrations shown in figures and tables. DMSO final concentration was less than 0.25% v/v. After incubation for 4 h at 37 °C, the generated water insoluble formazan dye was dissolved by addition of 100 µl of 10% w/v SDS in 0.01 M HCl. The plates were further incubated overnight at 37 °C, and optical density (OD) of the wells was determined using a microplate reader (Labsystems Multiskan MS, Finlandia) at 570 nm. Under these conditions, the OD is directly proportional to the viable cell number in each well. All experiments were performed at least three times and data are shown as the means and their standard deviations from triplicate cultures.

#### 2.3. Prostaglandin E₂ measurement

The PGE₂ levels were measured in the supernatant of an infected RAW cells culture. The “Prostaglandin E2 EIA Kit – Monoclonal” (Cayman Chemical Co., USA) kit was used, according to the manufacturer’s instructions. RAW 264.7 cells were infected with *T. cruzi* Dm28 clone trypomastigotes. After 72 h post-infection, 50 µl supernatant were tested using this immunoassay.

#### 2.4. Nitric oxide measurement

NO synthesis was evaluated indirectly, through nitrite content in supernatants from *T. cruzi*-infected RAW 264.7 cultures. Griess’ reactive was used to measure colorimetrically the nitrite content at 570 nm. In each experiment, nitrite concentration was calculated from a NaNO₂ standard curve (Tsikas, 2007).

#### 2.5. Effect of drugs on infected RAW 264.7 cells

The effect of aspirin, nifurtimox and benznidazole on *T. cruzi*-infected RAW 264.7 cells was assessed by the number of trypomastigotes released to the culture supernatants. Twenty-four hours after infection, aspirin, nifurtimox, benznidazole treatments and their combinations were started at concentrations described in results. Every 24 h culture media was removed and fresh medium was added together with the drugs at the same concentration. To show the influence upon NOS and COX, aminoguanidine (5 mM) and PGE₂ (1 mM), respectively, were added in some experiments. Cell culture medium was harvested at the third day post treatment and centrifuged at 500 g during 5 min. Supernatants were discarded, pellets resuspended in 1 ml of fresh RPMI 1640 and trypomastigotes were counted using direct microscopy (Freire-de-Lima et al., 2000; Nunes et al., 1998).

#### 2.6. Statistical analysis

IC₅₀ and growth rate calculations were analyzed by non-linear regression. When necessary, data were also analyzed through two-way ANOVA. The drug combination treatments were analyzed by isobologram constructions through sum-of-squares plus Fisher test. Values of p equal or less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 5.0. All experiments were done in triplicate and results correspond to means ± SD from at least three independent experiments.

### 3. Results

#### 3.1. Aspirin decreases trypomastigote release to culture supernatants from *T. cruzi*-infected RAW 264.7 cells

At fourth day of RAW cells infection, trypomastigotes began to be released to the supernatants. Aspirin showed a concentration-dependent effect, with an IC₅₀ of 313.1 ± 36.0 µM (Table 1). This concentration is below the steady-state concentration (IC₅₀, 1.1–2.2 mM after oral administration of 3–4 g/day) found in humans treated with aspirin at high concentrations, as is the highest aspirin concentration used in other experiments (1 mM) (Anderson et al., 2002; Keystone et al., 1982). This result is in agreement with previous reports, showing that aspirin and other COX inhibitors decreased *T. cruzi* infection (Abdalla et al., 2008; Freire-de-Lima et al., 2000; Kubata et al., 2002; Michelin et al., 2005; Paiva et al., 2007).

#### 3.2. Aspirin improves the activity of nifurtimox and benznidazole upon infected RAW 264.7 cells in a synergistic way

As expected, nifurtimox and benznidazole had a concentration-dependent effect on trypomastigote release with an IC₅₀ of 0.083 µM and 0.336 µM, respectively (Table 1). The addition of a low concentration of aspirin (IC₁₂.₅ of aspirin) reduces the IC₅₀ of nifurtimox from 0.083 to 0.023 µM (reduction by 72.3%) while the IC₅₀ of benznidazole was reduced by 61.7%. In addition, when aspirin was combined with nifurtimox or benznidazole (at its IC₁₂.₅ concentrations), the IC₅₀ value for aspirin was decreased by 57.2% and 76.7%, respectively. The observed IC₅₀ value is significantly lower than that expected for the combinations if the interaction was additive (p < 0.002) (Table 1). The graphical representation of this interaction is shown in the isobolograms of Fig. 1. These isobolograms allow the comparison of theoretical additive and observed experimental effects. Combinatory indexes (Chou, 2006) equal, lower or larger than 1, correspond to additive, synergistic or antagonistic behavior, respectively. Combinatory indexes for aspirin and nifurtimox or benznidazole combinations are described in Table 1.

Neither aspirin, nifurtimox, benznidazole nor their combinations, at the highest trypanocidal concentrations, showed cytotoxicity against uninfected RAW 264.7 cells (data not shown).

#### 3.3. Aspirin effect on infected RAW cells is not dependent of PGE₂ synthesis inhibition

Trypanosoma cruzi-infected RAW 264.7 cells presented a significantly higher prostaglandin (PGE₂) production compared to uninfected RAW cells. ASA reverted prostaglandin levels to baseline in a dose–response way (Fig. 2A). Note that the maximal effect of ASA
over the PGE\textsubscript{2} synthesis is achieved at 0.25 mM, a dose–response fit of this data showed an IC\textsubscript{50} of 7.7 ± 2.54 \textmu M. PGE\textsubscript{2} was added to reverse the effect of ASA 0.25 mM. The lack of effect of PGE\textsubscript{2} over ASA treatment might indicate that PGE\textsubscript{2} is not involved in the ASA effect on infected cells (Fig. 2B).

### 3.4. Aspirin restores NO\textsuperscript{–} levels in T. cruzi-infected RAW 264.7 cells

The effect of aspirin upon NO\textsuperscript{–} production was evaluated by nitrite detection in supernatants from T. cruzi-infected RAW 264.7 cells by the Griess reaction. While T. cruzi infection decreases NO\textsuperscript{–} levels, aspirin was able to restore these levels, as shown in Fig. 2B.

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**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (\textmu M)</th>
<th>IC\textsubscript{50} of the combination (\textmu M)</th>
<th>Combinatory index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>313.1 ± 36.0</td>
<td>250</td>
<td>0.71</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>0.083 ± 0.005</td>
<td>0.066</td>
<td>0.61</td>
</tr>
<tr>
<td>Aspirin</td>
<td>313.1 ± 36.0</td>
<td>250</td>
<td>0.61</td>
</tr>
<tr>
<td>Benznidazole</td>
<td>0.34 ± 0.002</td>
<td>0.27</td>
<td>0.34</td>
</tr>
</tbody>
</table>

\* Theoretical IC\textsubscript{50} corresponds to the value predicted from isobolograms depicted in Fig. 1. Nifurtimox, aspirin and benznidazole were added at their estimated IC\textsubscript{12.5} concentrations. \(p\) values correspond to the comparisons between theoretical and observed IC\textsubscript{50} values through sum-of-squares plus F test. Combinatory index was calculated according to Chou (2006). Results are expressed as means ± SD from three independent experiments.
Macrophages were infected with compared with infected control (ASA 0 mM). (B) Effect of aminoguanidine (AMG) release (black bars) in aspirin treated Raw 264.7 cells infected with *T. cruzi* trypomastigotes. After 72 h nitrite levels in supernatant were determined by the Griess reaction. § p < 0.001 compared with uninfected control (RAW). \( p < 0.001 \) compared with infected control (ASA 0 mM). (B) Effect of aminoguanidine (AMG) upon trypomastigote release in aspirin-treated Raw 264.7 cells. Macrophages were infected with *T. cruzi* trypomastigotes, and after 72 h treatment with 1 mM aspirin (ASA), 5 mM AMG or ASA plus AMG trypomastigote release to supernatants was measured. Results are expressed as means ± SD from three independent experiments. \( p < 0.05; **p < 0.001 \) from Tukey post-test.

![Figure 3](image)

**Fig. 3.** Effect of aspirin upon nitrite production and trypomastigote release in *T. cruzi*-infected RAW 264.7 cells. (A) Nitrite levels (white bars) and trypomastigote release (black bars) in aspirin treated Raw 264.7 cells infected with *T. cruzi* trypomastigotes. After 72 h nitrite levels in supernatant were determined by the Griess reaction. § \( p < 0.001 \) compared with uninfected control (RAW). \( p < 0.001 \) compared with infected control (ASA 0 mM). (B) Effect of aminoguanidine (AMG) upon trypomastigote release in aspirin-treated Raw 264.7 cells. Macrophages were infected with *T. cruzi* trypomastigotes, and after 72 h treatment with 1 mM aspirin (ASA), 5 mM AMG or ASA plus AMG trypomastigote release to supernatants was measured. Results are expressed as means ± SD from three independent experiments. \( p < 0.05; **p < 0.001 \) from Tukey post-test.

Attention can be drawn to the low reported IC\(50\) values of nifurtimox (0.0830 \(\mu\)M) and benznidazole (0.336 \(\mu\)M). However, this might be due to the low parasite density used in the experiments.

In previous experiments carried out on VERO cells (Faundez et al., 2005), parasite density was an order of magnitude higher and the highest concentration used for nifurtimox and benznidazole was 1 \(\mu\)M. Nevertheless, this situation is not an obstacle to perform synergism studies with other drugs.

Herein, the relationship of aspirin with classic antichagasic therapy is further substantiated. Thus, the IC\(50\) value of aspirin in infected macrophages is several folds higher than those determined for nifurtimox and benznidazole (Table 1). However, from the isobolographic analysis, we can conclude that aspirin acts synergistically with these classical drugs. In fact, the IC\(50\) of both nifurtimox and benznidazole, decreased in average by 67% when combined with aspirin (Table 1). However, the observed synergy is not related to a direct effect of both drugs upon the same target or pathway, i.e. parasite viability. Instead, the combined effects of aspirin on the macrophage and the toxic effects of antichagasic drugs on the parasite are complementary mechanisms that explain the isobolographic result. This concept is in agreement with synergistic mechanisms reported for other drug combinations (Jia et al., 2009).

Fig. 2A shows that PGE\(_2\) production increases significantly in *T. cruzi* infected macrophages as compared with healthy macrophages. Thus, PGE\(_2\) synthesis inhibition using aspirin could improve macrophage response against *T. cruzi* infection. In fact, ASA reduces PGE\(_2\) levels in a dose–response way, with an IC\(50\) = 7.7 \(\mu\)M. This value is 40-fold lower than the ASA IC\(50\) observed on trypomastigote release experiments (IC\(50\) = 313 \(\mu\)M). Therefore, complete PGE\(_2\) synthesis inhibition is achieved at concentration levels that have no significant effect on trypomastigote release. Moreover, there is no evidence supporting that PGE\(_2\) itself could increase *T. cruzi* infection. We attempted to determine whether PGE\(_2\) is involved in the effect of ASA upon *T. cruzi* infected cells. We did not find reversion of ASA effect, even at PGE\(_2\) concentrations as high as 1 \(\mu\)M (Fig. 2B), indicating that PGE\(_2\) does not have a main role on ASA effect. Other COX inhibitors, such as indomethacin and meloxicam were tested, both inhibiting trypomastigote release (IC\(50\) = 97.6 ± 11.1 and 42.7 ± 0.36, respectively). These data suggest a pivotal function of COX in intracellular amastigote proliferation. However, there might be other prostaglandins playing key roles in parasite proliferation. Cardoni and Antunez (2004) showed that *T. cruzi* infected mice have increased levels of other COX metabolites, such as PGI\(_2\) and TXA\(_2\), yet their role on parasite infection is still unknown (Cardoni and Antunez, 2004).

*Trypanosoma cruzi* induces TGF-\(\beta\) and PGE\(_2\) production by macrophages, especially in the presence of apoptotic bodies (Freire-de-Lima et al., 2000). Thus, the parasite induces an anti-inflammatory response, attenuating macrophage activation and evading its antiparasitic activity. In macrophages exposed to apoptotic bodies, TGF-\(\beta\) increases PGE\(_2\) synthesis and decreases NO (Freire-de-Lima et al., 2006). Inhibition of COX activity may increase NO\(^{\text{d}}\) levels, thus restoring the antiparasitic activity of macrophages. Our results are in agreement with this proposal (Fig. 3A and B). It is important to note that all these experiments were performed without exposure to apoptotic bodies. Consequently, *T. cruzi* itself can suppress macrophage antiparasitic activity in an apoptosis mimicry fashion, through, perhaps, *T. cruzi* calreticulin–C1q interactions (Ferreira et al., 2004). Thus, macrophage antiparasitic activity restoration can be pharmacologically modulated.

Finally, in *T. cruzi* infected macrophages, COX is related to the increase of ornithine decarboxylase (ODC) activity (Freire-de-Lima et al., 2000), which might increase the polyamine content in macrophages. Since *T. cruzi* uses these polyamines to synthesize trypanothione, the inhibition of COX by ASA, indirectly contribute to

**4. Discussion**

Different studies have shown the effect of COX inhibitors, such as aspirin, on several *T. cruzi* infection models (Freire-de-Lima et al., 2000; Hidéko Tatakihara et al., 2008; Michelin et al., 2005; Pinge-Filho et al., 1999). However, COX inhibitors concentration-dependent responses and mechanisms affecting *T. cruzi* proliferation inside host cells have not been studied. Herein, we studied the improvement of the macrophage antiparasitic activity induced by aspirin, the synergy of this effect with the activity of the antichagasic drugs nifurtimox and benznidazole and, possible mechanisms involved in those effects.
decrease trypanothione synthesis in T. cruzi. This mechanism, together with the restoring of NO levels in macrophages through COX inhibition, could be involved in the activity of ASA on the macrophage response to T. cruzi infection.

In conclusion, the observed synergistic effect of aspirin with nifurtimox and benznidazole is explained by the capability of aspirin to improve the antiparasitic activity of macrophages.

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