Journal Pre-proofs

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PII: S0006-2952(21)00120-9
DOI: https://doi.org/10.1016/j.bcp.2021.114524
Reference: BCP 114524

To appear in: *Biochemical Pharmacology*

Received Date: 21 December 2020
Revised Date: 10 March 2021
Accepted Date: 11 March 2021

Please cite this article as: W.M. Valsecchi, J.M. Delfino, J. Santos, S.H. Fernández Villamil, Zoledronate repositioning as a potential trypanocidal drug. *Trypanosoma cruzi* HPRT an alternative target to be considered, *Biochemical Pharmacology* (2021), doi: https://doi.org/10.1016/j.bcp.2021.114524

Zoledronate repositioning as a potential trypanocidal drug.

Trypanosoma cruzi HPRT an alternative target to be considered.

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Running Head: Zoledronate repositioned against *T. cruzi*.

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ABSTRACT

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and affects 7 million people worldwide. Considering the side effects and drug resistance shown by current treatments, the development of new anti-Chagas therapies is an urgent need. *T. cruzi* hypoxanthine phosphoribosyltransferase (TcHPRT), the key enzyme of the purine salvage pathway, is essential for the survival of trypanosomatids. Previously, we assessed the inhibitory effect of different bisphosphonates (BPs), HPRT substrate analogues, on the activity of the isolated enzyme. BPs are used as a treatment for bone diseases and growth inhibition studies on *T. cruzi* have associated BPs action with the farnesyl diphosphate synthase inhibition. Here, we demonstrated significant growth inhibition of epimastigotes in the presence of BPs and a strong correlation with our previous results on the isolated TcHPRT, suggesting this enzyme as a possible and important target for these drugs. We also found that the parasites exhibited a delay of S phase in the presence of zoledronate pointing out enzymes involved in the cell cycle, such as TcHPRT, as intracellular targets. Moreover, we validated that micromolar concentrations of zoledronate are capable to interfere with the progression of cell infection by this parasite. Altogether, our findings allow us to propose the repositioning of zoledronate as a promising candidate against Chagas disease and TcHPRT as a new target for future rational design of antiparasitic drugs.

Keywords: hypoxanthine phosphoribosyltransferase (HPRT), *Trypanosoma cruzi*, growth inhibition, bisphosphonates, zoledronate.

Abbreviations: *Trypanosoma cruzi* hypoxanthine phosphoribosyltransferase (TcHPRT), human HPRT (HsHPRT), Bisphosphonate (BP).

1. INTRODUCTION

Chagas disease is a neglected disease caused by the protozoan parasite *Trypanosoma cruzi* that affects approximately 7 million people worldwide, mainly in endemic areas of 21 continental Latin American countries (WHO) (https://www.who.int/chagas/epidemiology/en/), although cases have also been reported in non-endemic areas. Currently, approved treatments for Chagas disease are based on nifurtimox and benznidazole (BZN), available since 1965 and 1971, respectively. Both drugs show a high variability in the efficacy, nifurtimox has been demonstrated to cause adverse effects in long-term therapies, while BZN has been related to toxicity and drug resistance [1-3]. Therefore, the development of more effective and better-tolerated new anti-Chagas drugs is an urgent need.

Nucleotide synthesis in parasitic protozoa only occurs by the recovery pathway which requires a key enzyme called hypoxanthine phosphoribosyltransferase (HPRT) [4] (Figure 1). Considering that *T. cruzi* has no alternative pathways for GMP and IMP production than the salvage route, it is expected that inhibitors of TcHPRT should prevent its growth by blocking the synthesis of their DNA/RNA, a strategy previously used for the development of chemotherapeutics that prevent the growth and proliferation of parasites [5]. HPRT activity was reported as essential for *Leishmania donovani*, *Plasmodium falciparum*, *T. cruzi* and *Mycobacterium tuberculosis* [6-9]. Given these facts, *T. cruzi* HPRT (TcHPRT) has been proposed as a prime target for drugs aimed at treating parasitic diseases (*see TDRtargets Database*, *http://tdrtargets.org*).

Regarding the design of inhibitory molecules that show selectivity for TcHPRT, an approach is to design molecules with high mimicry to the transition state of the reaction catalyzed [10]. With the aim of testing molecules similar in structure to PRPP, we previously carried out the study of the effect produced on TcHPRT activity of a set of bisphosphonates

(BPs), molecules that emulate the pyrophosphate moiety of PRPP [11, 12]. In the past, several BPs have been established as therapeutic agents for the prevention of skeletal complications connected with multiple myeloma or bone metastases [13]. It was also demonstrated that BPs reduce the risk of fractures and increase bone mineral density so that they are widely used for the treatment of menopausal osteoporosis in women, osteoporosis induced by glucocorticoids, and imperfect osteogenesis in children [14, 15]. Currently, the BP named zoledronate has been matter under study in principal areas of medical science such as breast cancer [16, 17] and bone marrow lesions [18, 19].

Bisphosphonates accumulate in the *T. cruzi* acidocalcisomes and can inhibit enzymes involved in inorganic and organic pyrophosphate reactions such as farnesyl pyrophosphate synthase (FPPS), nevertheless other potential target molecules have not been discarded [3]. Here, we examine the effect of a select group of BPs on *T. cruzi* growth and postulate TcHPRT as a possible target for these drugs.

Drug repositioning involves finding novel indications for approved drugs, giving new answers to old problems; using drugs already established for human use greatly shortens development timeframes saving time and money, since these compounds have shown proven toxicological and pharmacokinetic profiles, and the evaluation phases have already been approved. In this regard, we advance the argument of repositioning zoledronate as a candidate drug against Chagas disease.

2. MATERIALS AND METHODS

2.1. Culture media and reagents

T. cruzi epimastigotes were cultured in LIT (**L**iver **I**nfusion **T**ryptose): (5 g L^{-1} liver infusion, 5 g L^{-1} bacto-tryptose, 68 mM NaCl, 5.3 mM KCl, 22 mM Na_2HPO_4, 0.2 % (W/V) glucose, and 0.002 % (W/V) hemin) supplemented with 10 % fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin) for 7 days at 28 °C. FBS was from

Natocor, Argentina. Bacto-tryptose and liver infusion were from Difco Laboratories, Detroit, MI. Vero cells were cultured in MEM (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 3 % FBS, at 37 °C and 5 % CO_2 atmosphere. The set of BPs used in this work was provided by GADOR S.A., Buenos Aires. Other reagents were from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Screening of inhibitors

T. cruzi epimastigote *Tulahuen strain* was grown at 28 ºC in LIT medium supplemented with 10 % FBS, 100 µg mL^{-1} streptomycin and 100 U mL^{-1} penicillin for 4 days (exponential growth). Parasites (10^6 mL^{-1}, 100 µl) were placed in 96-well sterile plates in the presence of the corresponding BP solution. Control samples were grown in LIT in the absence of BP. Optical density ($OD_{600\ nm}$) of the cultures was determined for 4 days to follow cell viability. All conditions were assayed in triplicates in each of 14 experiences. The significance of the results was analyzed using the Bonferroni test. IC50 values were obtained by non-linear regression logistic functions, using GraphPad Prism 6.1 for Windows. Results are shown as mean ± standard deviation (SD).

TcHPRT activity was determined in a previous work [11], showing a biphasic behavior in the presence of BPs. Two hyperbola equations were fitted to the experimental data by a nonlinear regression procedure using *OriginPro 2017* to obtain the $K_{0.5}$ values. Results are expressed as mean ± SD.

2.3. Cell cycle analysis on epimastigotes cultured in the presence of zoledronate

T. cruzi epimastigotes were cultured in LIT medium with or without 1.5 mM zoledronate. After 4 days of growth, cells were fixed with 70 % ethanol, stained with propidium iodide (Sigma-Aldrich, St. Louis, MO, US) and then analyzed by flow cytometry.

2.4. Hydroxyurea-induced synchronization and flow cytometry analysis

T. cruzi epimastigotes in exponential growth were diluted in LIT medium up to $2\ 10^6\ mL^{-1}$ to be synchronized at G1 phase with 20 mM hydroxyurea (Sigma-Aldrich, St. Louis, MO, US) for 18 h. Synchronized epimastigotes were washed 3 times with cold PBS and resuspended in LIT medium supplemented with or without 0.8 mM zoledronate. Both cultures were incubated at 28 ºC and 1 mL samples were collected every 2 h during 24 h. Cell cycle was analyzed by flow cytometry using propidium iodide and the samples previously fixed with 70 % cold ethanol.

Data analysis was made with FlowJo X10 software. Briefly, after a 10000-events collection, cells with the expected size and complexity were selected to make the histogram. Phases G1, S and G2/M were defined over the histogram generated and those settings were fixed all over the analysis. Finally, control and treatment histograms were superimposed and the percentage of epimastigotes in each phase was calculated. It is worth noting that the experiment was made by triplicate with similar results.

2.5. β-galactosidase assay

The assay was performed on Vero cells (ATCC® CCL-81™) in a p96 plate. Cells, (10^4 seeded per well) were infected in MOI (multiplicity of infection) 10:1 with trypomastigotes carrying the gene for β-galactosidase [20]. After 24 h incubation period, fresh media plus each study drug was added to infected monolayers. At 96 h p.i. (post-infection), cell culture media was removed and Tulahuen β-galactosidase parasites were lysed in 100 µL lysis buffer (25 mM Tris-HCl, pH 7.8; 2 mM EDTA; 1 % Triton X100; 10 % glycerol; 2 mM DTT), a condition in which cells were incubated for 15 min at 37 °C. Then, 100 µL of reaction buffer (200 mM NaH_2PO_4/Na_2HPO_4, pH 7.0; 2 mM $MgCl_2$; 100 µM β-mercaptoethanol; 1.33 mg mL^{-1} o-nitrophenyl-β-D-galactopyranoside (ONPG)) was added and the absorbance increase, as a consequence of ONPG hydrolysis, was detected at 420 nm

in a Synergy HTX multi-mode microplate reader during the following 30, 45, and 60 min. All conditions were assayed in triplicates of five independent experiences. IC50 values were obtained by non-linear regression logistic functions, using GraphPad Prism 6.1. Results are expressed as mean ± SD. All reagents were from (Sigma-Aldrich, St. Louis, MO, US).

2.6. Alamar blue assay

The test was performed on Vero cells in a p96 plate. Cells (10^4 per well) were seeded in MEM (100 µL) supplemented with 10 % FBS. Cells were incubated for 24 h and the media was replaced by MEM-3 % FBS with the study drugs. Cells were incubated in this condition for 72 h at 37 °C and 5 % CO_2 atmosphere. Then, the medium was replaced by fresh medium and resazurin (Sigma-Aldrich, St. Louis, MO, US) solution (10 µg mL^{-1} final concentration). Resazurin shows an increased fluorescence in its reduced form. After 30 min fluorescence was measured (excitation at 530 nm and emission at 590 nm).

3. RESULTS

3.1. *In vitro* trypanocidal activity of BPs

Previously we reported that BP derivatives alendronate, ibandronate, lidadronate, olpadronate, pamidronate, and zoledronate, show inhibitory effect on the recombinant TcHPRT [11]. These BPs display a characteristic biphasic behavior on enzymatic activity, showing activation at low concentrations whereas behaving as inhibitors at high concentrations. Considering that ibandronate had turned out to be the best inhibitor and olpadronate, the best activator, we tested both on the human variant HsHPRT. At a concentration as high as 500 µM, both ibandronate and olpadronate scarcely inhibit HsHPRT (21.2 and 4.1 % respectively) as compared to the control sample. This result opens the possibility of considering these and related BPs as trypanocidal agents. In this regard, we evaluated the response of epimastigotes to BPs and analyzed the culture growth rate relative to the control up to day 4 of growth. Concentrations were selected as multiples of the IC50 for

purified recombinant TcHPRT. All concentrations assayed inhibited cell growth, an effect that becomes significant since day 2 (Figure 2). Zoledronate and ibandronate proved to be the best inhibitors. Nevertheless, statistical analysis showed no significant differences for 1.5 mM zoledronate between days 1 and 4, which indicates that cell growth is totally arrested after 24 h exposure to the drug. The same analysis in the case of ibandronate shows that the growth is inhibited since day 3 at the same concentration, suggesting a relatively greater potency for zoledronate than ibandronate. At similar concentrations, the other BPs assayed show much reduced ability to inhibit the growth of epimastigotes, with olpadronate and pamidronate showing an intermediate effect (reaching a plateau at about 50 %). Both alendronate and lidadronate showed only roughly 20 % of growth inhibition under similar conditions (~1.2 mM).

We calculated the BP concentrations required to inhibit 50 % of TcHPRT activity on the purified protein ($K_{0.5}$) and to inhibit 50 % of parasite growth at day 4 (IC50). The inhibitory potency in each case was (zoledronate/ibandronate) >> (pamidronate/olpadronate) > (lidadronate/alendronate) (Table 1). This outcome suggests a correlation in the inhibitory effect seen on TcHPRT and the epimastigotes growth inhibition. Moreover, these results also suggest that these BPs are efficiently incorporated into parasites, which is important when considering the multiplicity of factors that would arbitrate their transport.

3.2. BPs affect *T. cruzi* proliferation and cell infection

To further study the effect of BPs on the intracellular replicative form amastigote, we seeded Vero cells on 96-well plates and β-galactosidase-expressing trypomastigotes were allowed to remain in contact with cells for 24 h. After that period, medium was removed and replaced with fresh medium added with the corresponding inhibitors. Parasite counts were then determined spectrophotometrically by measuring the product of the enzymatic reaction 96 h p.i. A graphical representation of the infection schedule is shown in Figure 3. The

compounds were tested at different concentrations ranging from 0–2.5 mM. It should be noted that in the experimental design, we excluded pamidronate and olpadronate, since these are the BPs showing intermediate effects both on epimastigotes and on the isolated enzyme. The result for each condition is expressed relative to the value obtained for the infection in the absence of inhibitors. In order to test the possible toxicity of these compounds on host cells, we evaluated the viability of the Vero cells by using the Alamar Blue method. Zoledronate, ibandronate and alendronate led to a decrease in the measured β-galactosidase activity, indicating a decrease in the infection levels (Figure 3). These results also indicate that BPs manage to cross the membrane of Vero cells, and efficiently reach their target molecules. While the same concentration of zoledronate and alendronate (50 µM) causes a similar decrease in the infection level (~85 %), alendronate is too toxic for the host cell (~75 % toxicity). By contrast, lidadronate is not toxic to Vero cells, albeit it is also well tolerated by parasites in the same range of concentrations.

Thereafter, we estimated the selectivity index (SI), a metric that relates the IC50 for Vero cells, with the IC50 for the amastigotes (Table 2), thus BPs with higher SI will be considered the best inhibitors. As we had previously interpreted, these results showed antiparasitic potencies in the following order: zoledronate > ibandronate > alendronate > lidadronate. At similar low µM IC50 values, the SI for zoledronate (55.5) compares with advantage to that corresponding to BZN (20.1) [21], the most widely used drug for the treatment of Chagas disease, placing the former as an interesting drug candidate.

3.3. Zoledronate impairs epimastigote cell cycle

As *T. cruzi* has no alternative pathways to bypass HPRT for purine nucleotides production, it is expected that inhibitors of TcHPRT should block the synthesis of DNA/RNA, impairing the cell cycle. With the aim of studying whether the cell cycle is arrested at a particular phase, we cultured *T. cruzi* epimastigotes for 4 days in the absence or

in the presence of 1.5 mM of zoledronate, and we determined then the populations in each cell cycle phase by flow cytometry (Figure 4). Results showed alterations in the pattern observed when parasites were grown in the presence of zoledronate (Figure 4C). It is possible that this difference lies in the difficulty for parasites to complete the cell cycle in the presence of this BP.

Considering these observations, we challenged a previously synchronized culture using hydroxyurea (HU) —an inhibitor of DNA synthesis— allowing next the parasites to complete their cycle in the absence or in the presence of zoledronate. We choose to test 0.8 mM of drug since this concentration was the lowest exhibiting the maximum inhibitory effect at day 4 of growth. While non-synchronized cells show their population distributed in similar proportions among the three phases of the cycle, we found that synchronized epimastigotes are mainly at G1/S boundary, the valley of the S region is very marked and there are very few cells in the G2/M phase. Besides, control epimastigote cell cycle was completed in 18-20 h, in agreement with previous reports [22]. Results depicted as histograms in Figure 5A show that both cultures untreated and treated with 0.8 mM zoledronate progressed similarly through G1. However, it is possible to observe a delay in the cell cycle starting at 6h after HU removal, indicating that the S phase proceeds more slowly in treated cells. The observed lag is accentuated as time goes by, and therefore cells exposed to zoledronate require more time to return to the G1 phase. Note that between 14 and 18 h after HU removal most control cells culminate the S phase, while in the culture exposed to zoledronate still 20 % of cells remain in this phase decreasing only after 22 h (Figure 5C). This delay explains why at 22 h post HU removal, twice this population of cells does not complete mitosis, as compared to control cells (39.6 % cells in G2/M for zoledronate vs. 19.9 % for control) (Figure 5B and D).

All in all, these observations indicate the difficulty in the progression through the cell cycle of the culture exposed to the drug and suggest an effect of zoledronate on enzymes

related to the cell cycle. Accordingly, it is possible to consider TcHPRT among the targets of this BP.

4. DISCUSSION

Many efforts to identify new targets for Chagas disease are constantly pursued, as recently reviewed [23, 24]. However, since nifurtimox and BZN were discovered no other drug was introduced into the market and they are efficient only in the acute phase [1]. Regarding the development of efficient and well-tolerated drugs against the parasite, repositioning of drugs used for other pathologies is cost-effective and a strategy recommended by the World Health Organization (WHO) to tackle neglected diseases like Chagas.

BPs have been available for the treatment of osteoporosis, Paget's disease, the hypercalcemia of malignancy, and bone metastases derived from various cancer types. We reported that BPs inhibit TcHPRT, an essential enzyme for *T. cruzi* and rationalized the structural principles underlying the inhibitory effects observed by each BP by docking *in silico* [11]. The BPs studied here show inhibitory effect on the proliferation of the parasite, revealing zoledronate the higher relative potency. This result agrees with our previous statement on the inhibitory effects on TcHPRT of these BPs, where we suggested that the higher inhibitory power of zoledronate could be due to the presence of a diffuse positive charge in the aromatic ring of imidazole, a feature resembling the transition state of the pyrophosphorolysis and condensation reactions [11]. Further co-crystallization assays of TcHPRT and other HPRTs with BPs might shed light on the differences in the mechanism of action of zoledronate in those enzymes.

Looking for inhibitory compounds that mimic substrates or products of key enzymes for parasite survival, molecules acting on several target enzymes could be interesting candidates, since compensating for their absence would be more difficult for the cell. On this matter, it

has been described that TcFPPS is inhibited by BPs [25, 26], and Demoro *et al.* stated that the tested compounds produce a decrease in the proliferation of *T. cruzi* amastigotes as a consequence of a multi-target effect [27]. Such multi-target action, probably involves TcHPRT, an option that has not been considered so far.

Zoledronate (one of the two compounds with greater inhibitory power on recombinant TcHPRT) was the best growth inhibitor for both the epimastigote and amastigote forms of the parasite. To shed light on the intracellular mechanism of action of zoledronate, we studied its effect on epimastigotes cell cycle. Since TcHPRT is essential for purine nucleotides production, it is expected that inhibitors of TcHPRT block the parasite DNA/RNA synthesis. Our results indicate that zoledronate makes difficult the transit through the S phase of the cell cycle, which could suppose an action on enzymes related to DNA synthesis such as TcHPRT. The correlation between our results on the recombinant TcHPRT and the replicative forms of the parasite strongly suggests that the enzyme pointed out could be one of the most important targets.

Among other BPs, zoledronate has also been tested as a potential agent against *Leishmania tarentolae* [28], *Trypanosoma brucei* [29], *Plasmodium falciparum* and *Entamoeba histolytica* [30], and lipophilic analogs of zoledronate and risedronate were postulated as potent antimalarial drugs [31]. A double-hit strategy combining inhibitors of host and parasite pathways was proposed as a novel approach against toxoplasmosis by using zoledronic acid and atorvastatin [32]. These results support zoledronate as a promising antiprotozoal candidate.

Recently, a meta-analysis suggests that alendronate and zoledronate are the BPs of choice for the treatment of osteoporosis [33], and zoledronate is the most extensively used BP in cancer therapy for preventing skeletal complications in patients with bone metastases. New formulations of alendronate or zoledronate by encapsulation in liposomes or nanoparticles are

being investigated to increase their effectiveness and reduce the doses used [34]. On the other hand, intravenous administration of zoledronate [35] and the combination of zoledronate with other drugs [36, 37] are being tested in order to reduce its adverse effects. These studies can be inspiring for stimulating research on the treatment of other diseases.

An open-label pharmacokinetic and pharmacodynamic study of zoledronic acid performed in patients who received 4 mg dose displays plasmatic concentrations of approximately 320 ng mL^{-1} [38]. These serum levels are in the same order as zoledronate IC50 for the intracellular form of the parasite. Moreover, we determined SI values for zoledronate > 50, agreeing with the highest inhibitory effect on the parasite and low cytotoxicity observed. This is particularly significant since a SI of > 50 is considered adequate for trypanocidal drugs, which reinforce the repurposing of zoledronate as a possible anti-Chagas agent [39-41]. Regarding the drug repositioning strategy, nowadays the combined therapy of drugs with different mechanisms of action is also considered an adequate strategy for achieving a synergistic effect and delaying or overcoming the appearance of drug resistance [27, 42, 43]. Likewise, the combination of BZN with new drugs is also an alternative under study [21, 44, 45].

To sum up, for its essential role TcHPRT has been suggested as a potential target against *T. cruzi*. Our results show that the inhibition of TcHPRT effectively affects the parasite cell cycle, leading to a decrease in parasite growth and impairment in the progression of cell infection. Zoledronate, a BP with therapeutic uses in constant updating, appears as a promising candidate for drug repurposing as an anti-Chagas drug due to its effects on TcHPRT, although this argument does not rule out the involvement of other molecular targets. These results encourage the scientific community to further investigate zoledronate to facilitate their use for the treatment of this trypanosomiasis and other neglected diseases.

ACKNOWLEDGMENTS

We thank Dra. Miriam Dziubecki and Dr. Emilio Roldán from GADOR SA, Buenos Aires for providing the set of BPs used in this work. We also thank Rodrigo G. Ducati, PhD (Albert Einstein College of Medicine, USA), for gently providing us with HsHPRT, William A. Agudelo (Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá D.C., Colombia), for his comments and suggestions, and Salomé C. Vilchez Larrea, PhD (INGEBI, Argentina) for her collaboration in cell culture and β-galactosidase-trypomastigotes production. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica PICT 2015-0898 and Universidad de Buenos Aires.

CREDIT AUTHOR STATEMENT

Wanda M. Valsecchi: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Writing - Original Draft; Writing - Review & Editing; Visualization. **José María Delfino**: Conceptualization; Writing - Review & Editing; Visualization. **Javier Santos**: Conceptualization; Writing - Review & Editing; Visualization. **Silvia H. Fernández Villamil**: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Resources; Writing - Original Draft; Writing - Review & Editing; Visualization; Supervision; Project administration; Funding acquisition.

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Purine recovery pathway. HPRT catalyzes the transfer of ribose 1-phosphate from phosphoribosyl pyrophosphate (PRPP) to hypoxanthine (Hx) or guanine bases, yielding IMP or GMP, respectively, and pyrophosphate (PPi) (highlighted in red). Besides, HPRT can salvage guanine, and in some cases xanthine. APRT, adenine phosphoribosyltransferase, and XPRT, xanthine phosphoribosyltransferase. The *de novo* pathway (involving several enzymatic steps) to generate IMP from PRPP, carbon dioxide (CO_2), amino acids, and tetrahydrofolate derivates (THF) is also indicated.

Figure 2. Epimastigotes cultured in the presence of BPs for 96 h. Each column represents the growth percentage ± SD. Bonferroni multiple analysis was used; in each group (*) $p \leq 0.0001$, (+) $p \leq 0.001$, (‡) $p \leq 0.01$ and (#) $p \leq 0.05$. The indicated statistical analyses were carried out for individual days, and referred to the control (black symbols) or to the highest concentration of each drug (red symbols).

Figure 3. ***T. cruzi* proliferation and cell infection.** The infection protocol scheme is shown on the top. The parasite count was estimated by absorbance at 420 nm according to the β-galactosidase activity protocol. Vero cells viability in each condition was estimated by Alamar blue assay. In all cases, data is presented as mean ± SD.

Figure 4. **Flow cytometry analysis of non-synchronized *T. cruzi* epimastigotes in the presence of zoledronate.** (A) Control epimastigotes sample; Ca and Cb are experimental duplicates. (B) Zoledronate exposed epimastigotes (1.5 mM); Za and Zb indicate experimental duplicates. (C) Ca and Za comparison. Population percentages are detailed in the insets.

Figure 5. Cell cycle phases of epimastigotes cultured in the absence or in the presence of zoledronate. Histograms belonging to control (red) and 0.8 mM zoledronate exposed (blue) cultures (A). To facilitate the comparison, population percentages corresponding to phase G1 (B), S (C), and G2/M (D) are shown at each time post HU-release.

CREDIT AUTHOR STATEMENT

Wanda M. Valsecchi: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Writing - Original Draft; Writing - Review & Editing; Visualization. **José María Delfino**: Conceptualization; Writing - Review & Editing; Visualization. **Javier Santos**: Conceptualization; Writing - Review & Editing; Visualization. **Silvia H. Fernández Villamil**: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Resources; Writing - Original Draft; Writing - Review & Editing; Visualization; Supervision; Project administration; Funding acquisition.

Table 1. Comparison between $K_{0.5}$ (TcHPRT) and IC50 (epimastigotes) values.

Bisphosphonate	$K_{0.5}$ (µM)	IC50 (µM)
Alendronate	>7000	>5000
Lidadronate	>7000	>5000
Pamidronate	197.8 ± 13.4	2857 ± 120
Olpadronate	193.1 ± 24.3	1640 ± 100
Zoledronate	162.3 ± 23.2	60.5 ± 10.1
Ibandronate	154.4 ± 17.3	87.4 ± 15.1

497

498

Table 2. IC 50 and Selectivity Index values for the selected compounds.

Compound	(IC50 µM)		SI
	Vero	*T. cruzi*	
BZN	82.8 ± 2.8	4.1 ± 0.4	**20.1**
Alendronate	18.9 ± 2.6	6.6 ± 0.2	**2.8**
Lidadronate	>2500	>2500	**-**
Zoledronate	322.2 ± 37.8	5.8 ± 0.3	**55.5**
Ibandronate	1199 ± 232	105.4 ± 15.6	**11.4**

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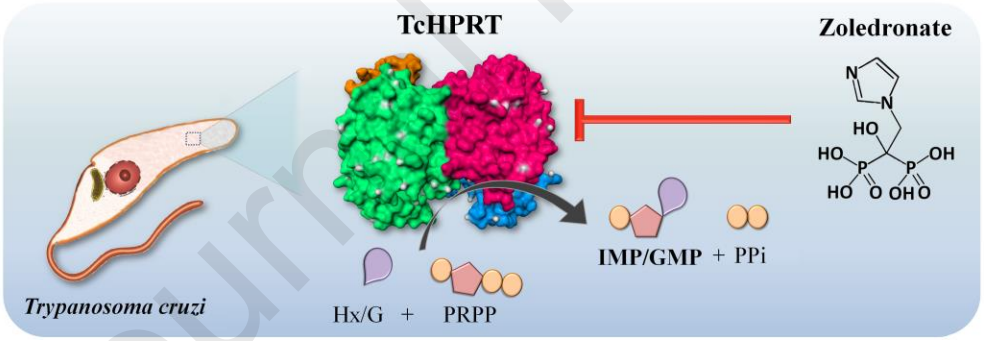

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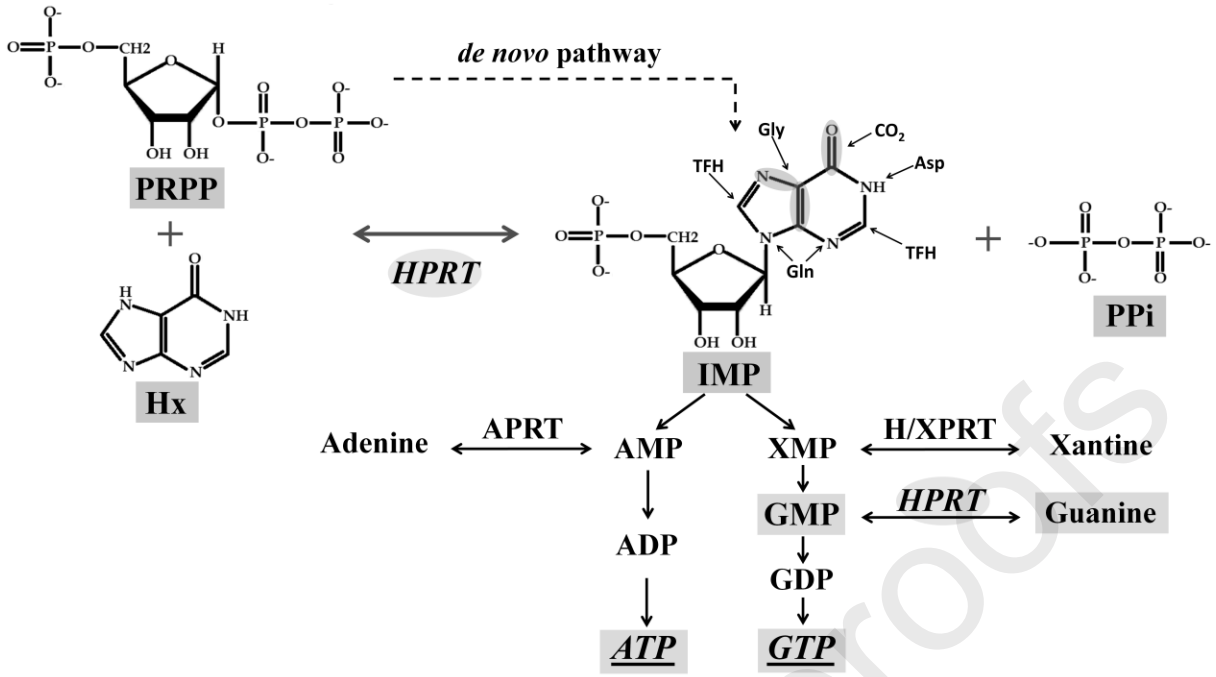

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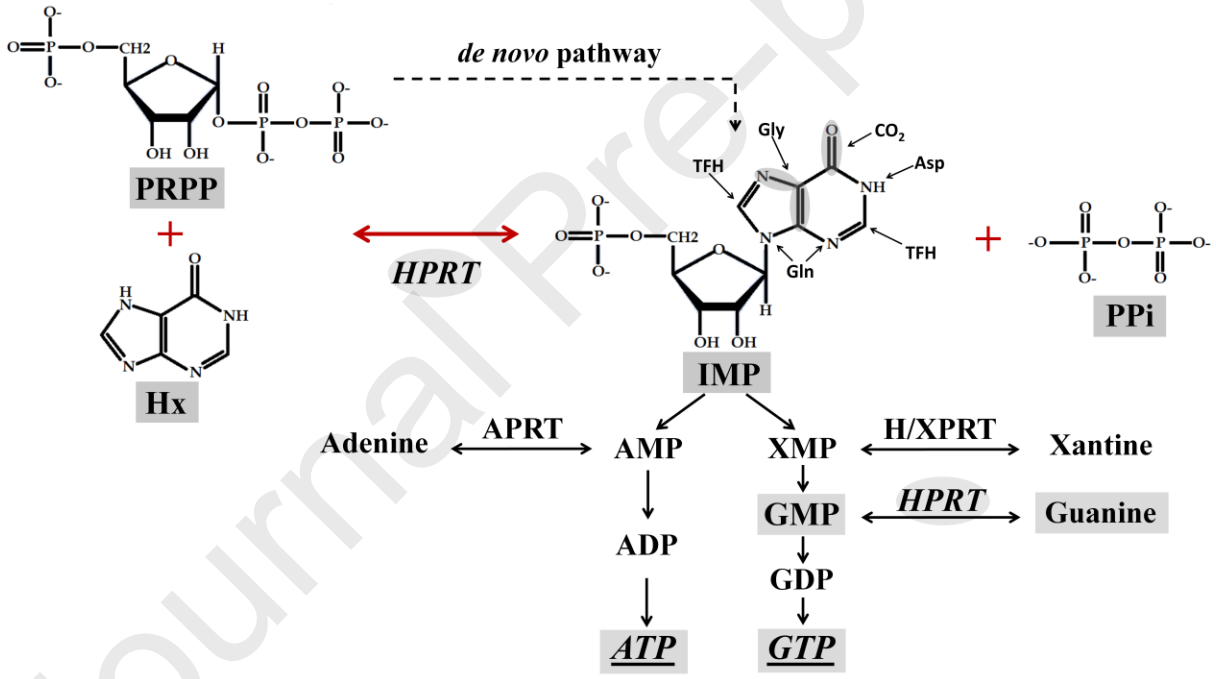

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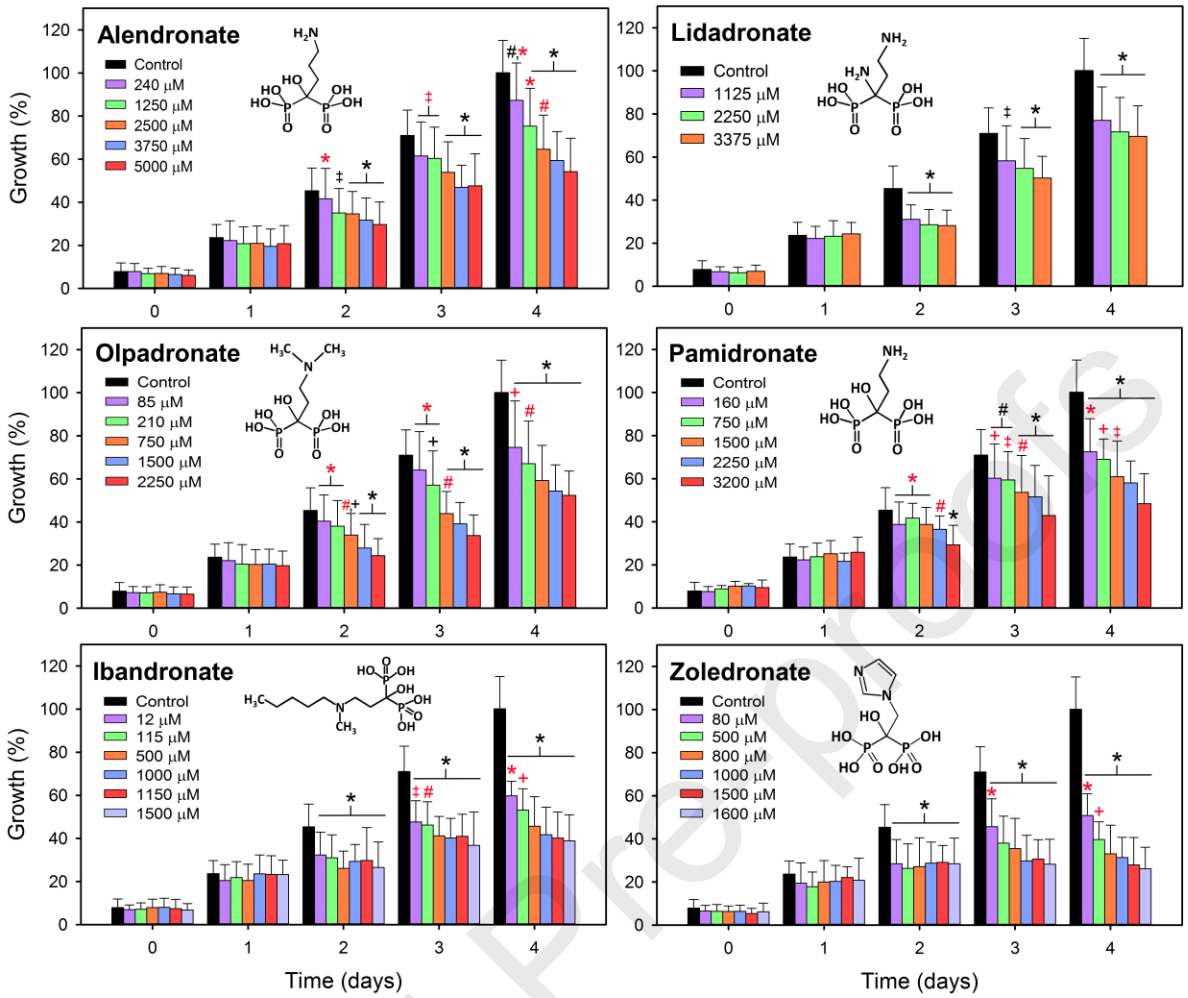

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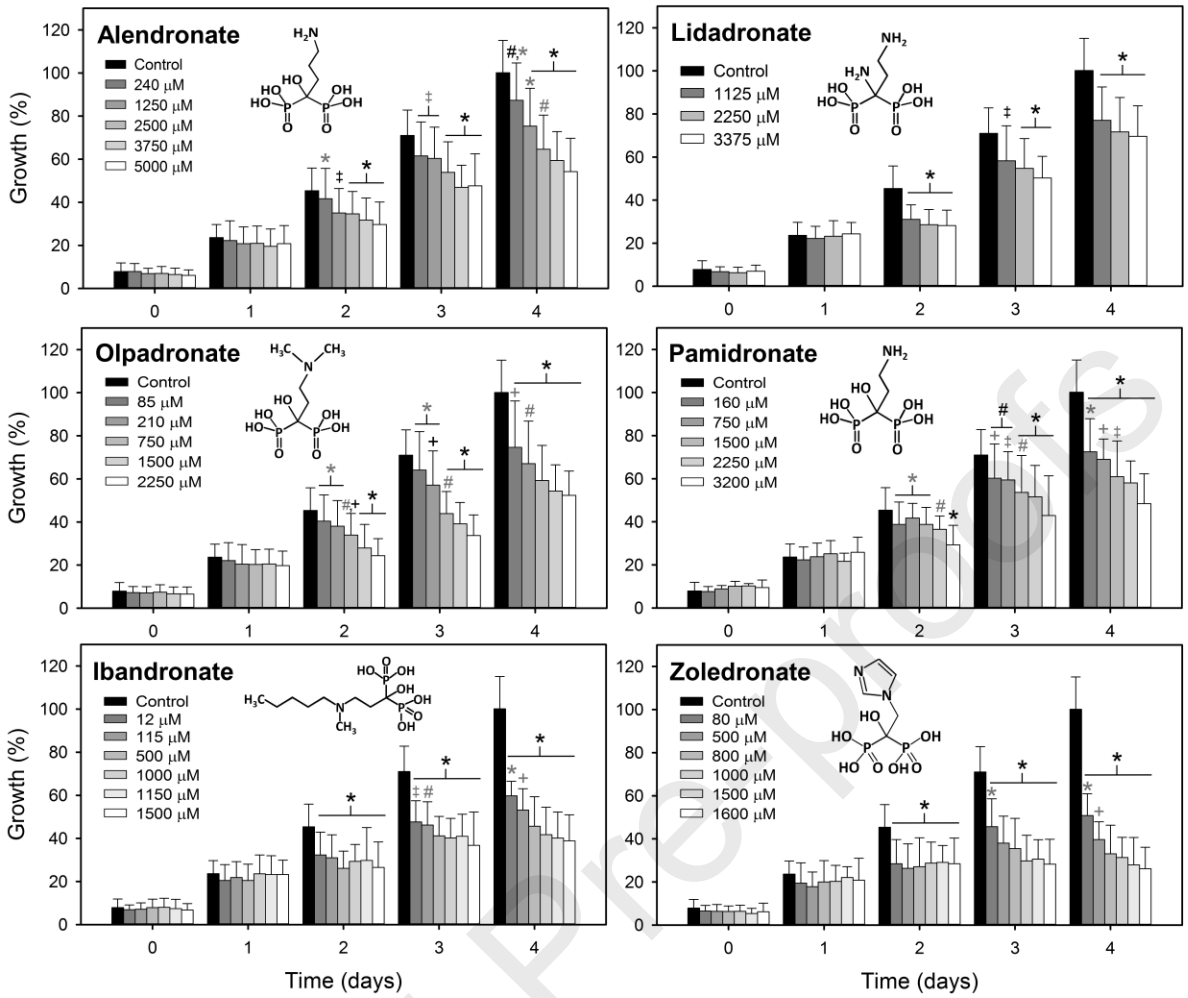

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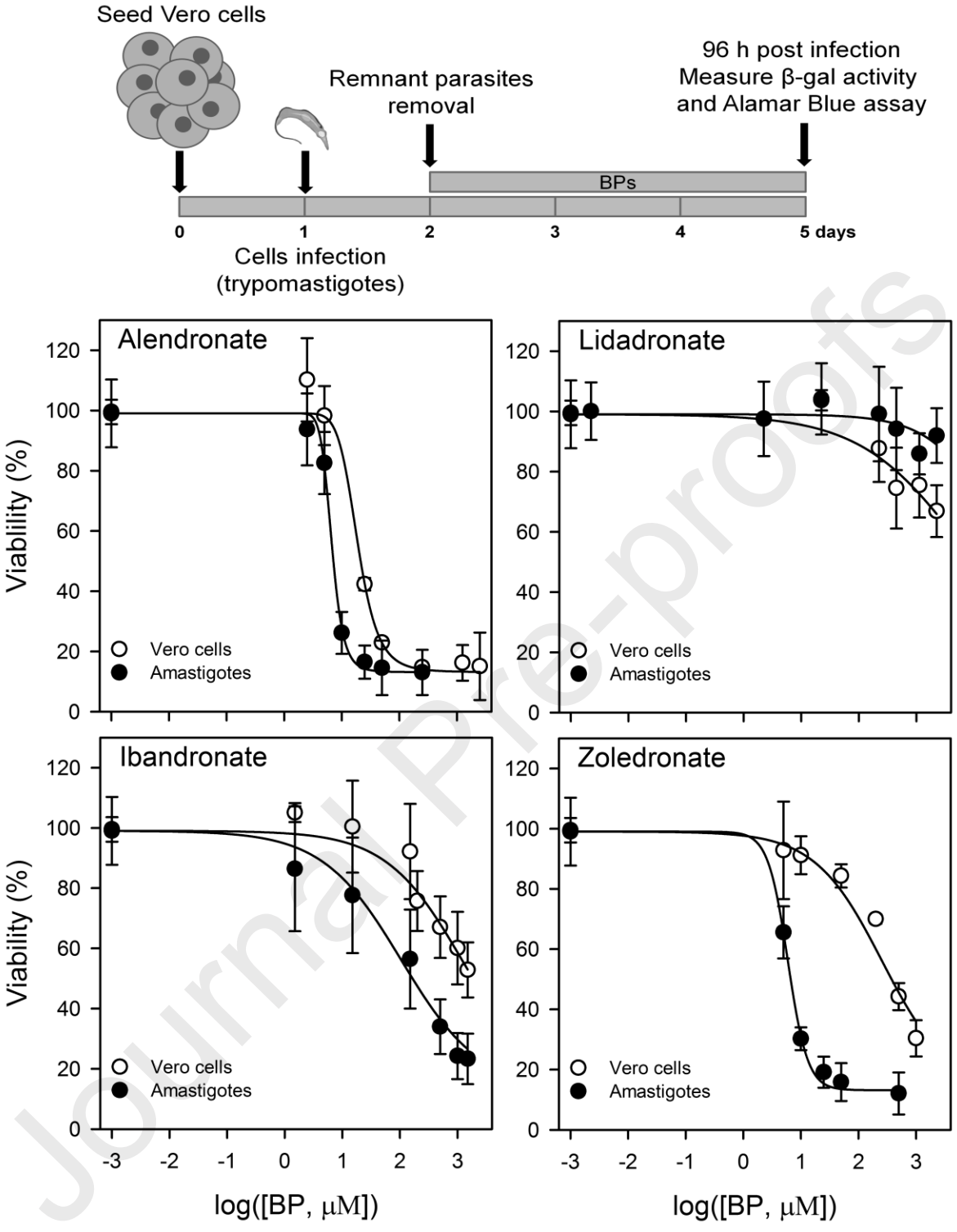

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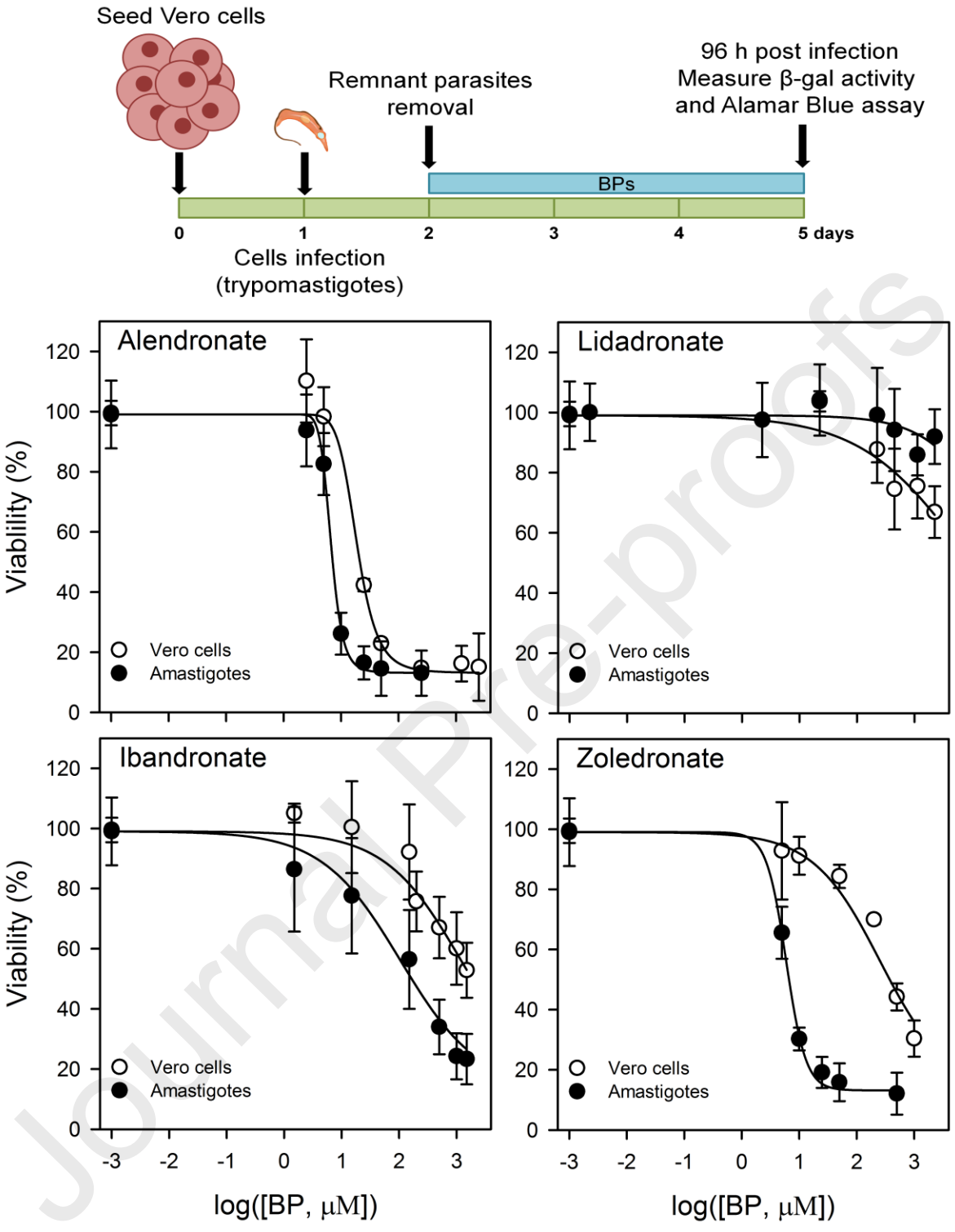

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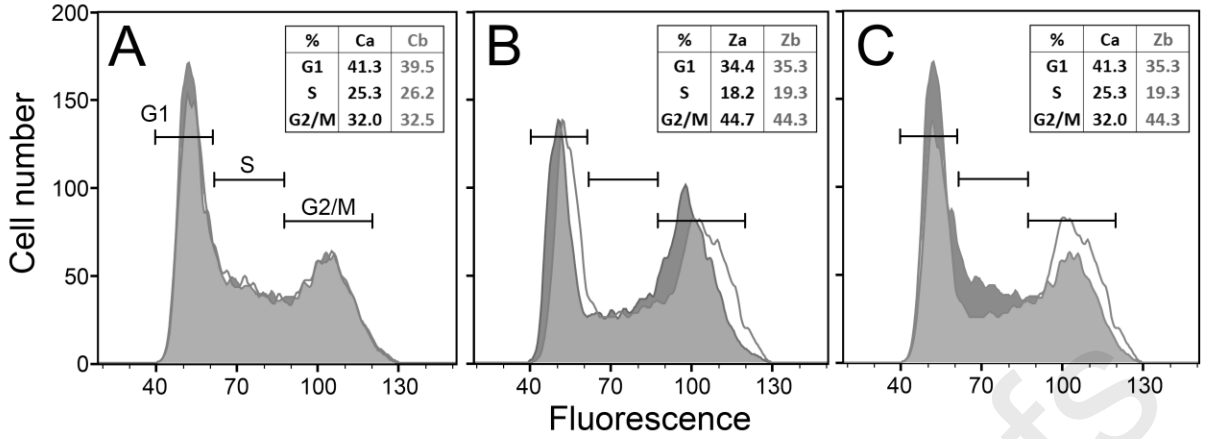

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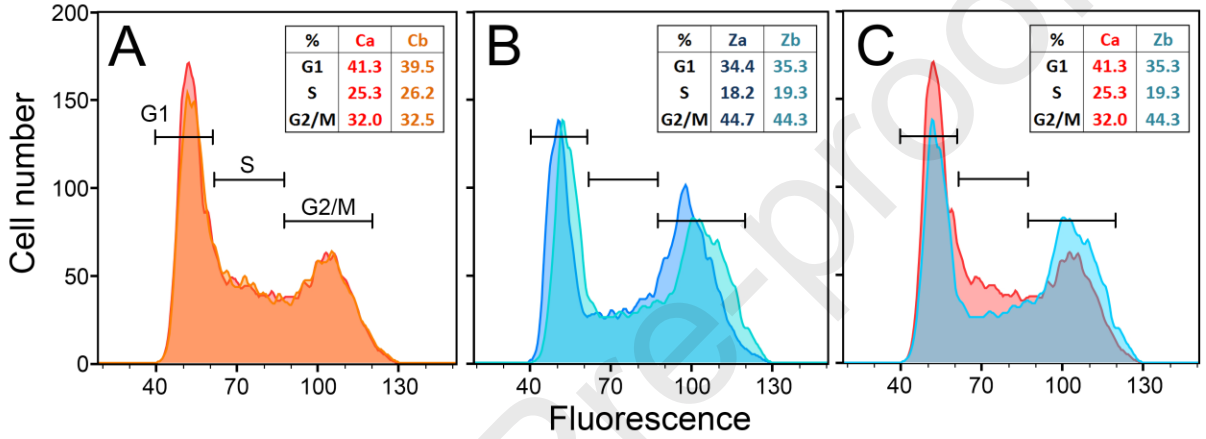

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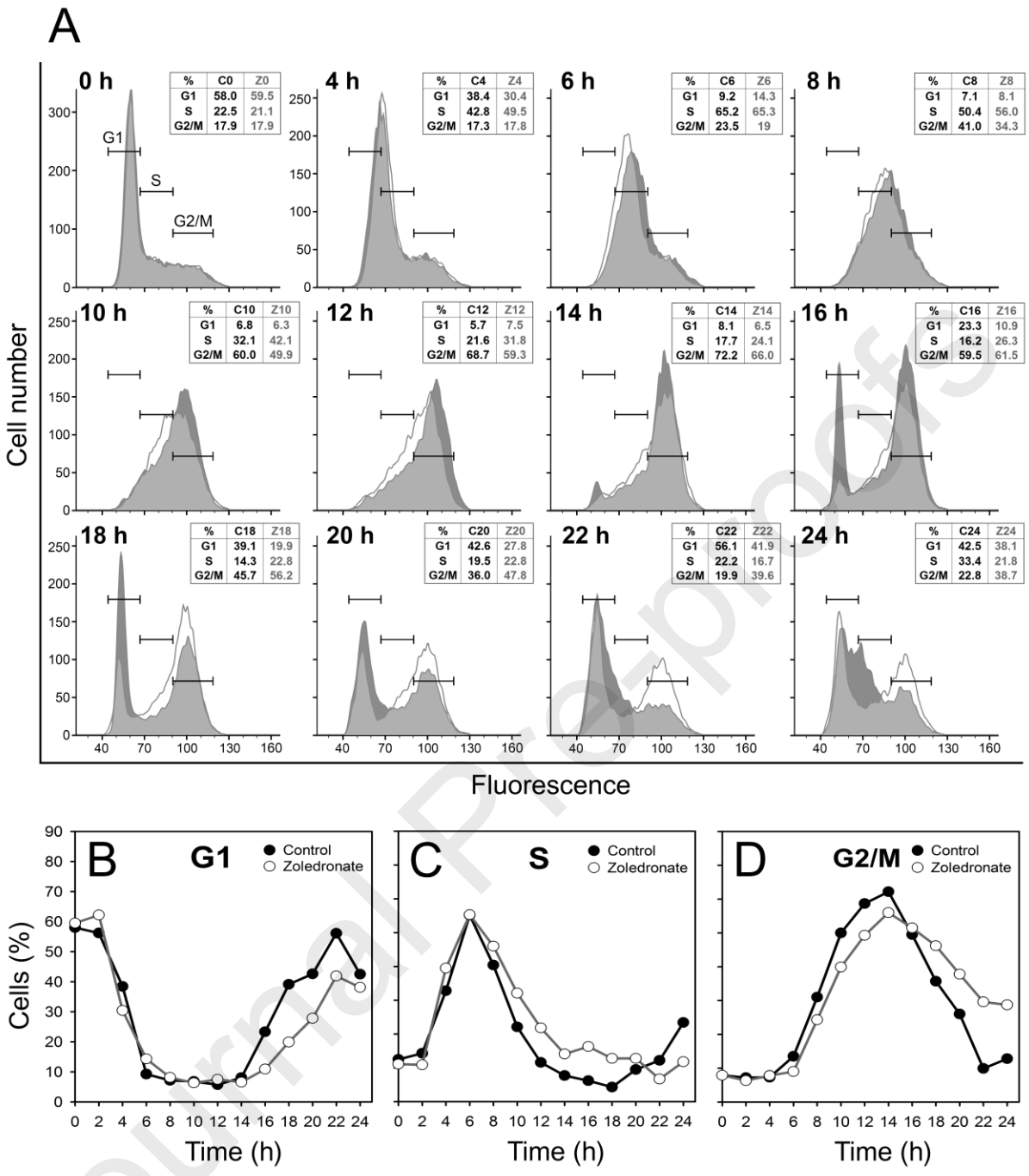

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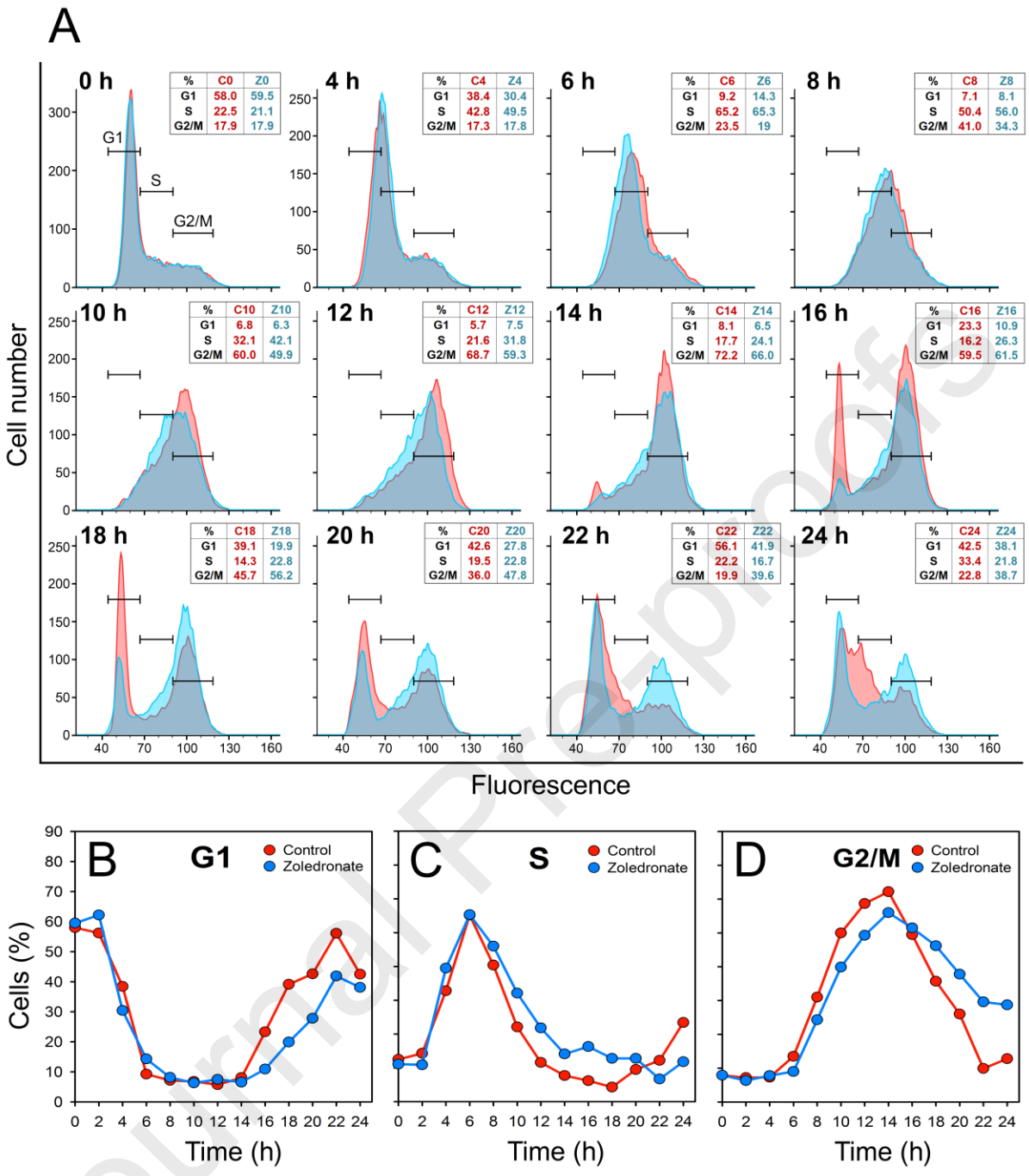

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