A new combined flow-cytometry-based assay reveals excellent activity against *Toxoplasma gondii* and low toxicity of new bisphosphonates *in vitro* and *in vivo*

Hend M. Shubar¹, Jan Patino Mayer¹, Werner Hopfenmüller² and Oliver Liesenfeld¹*

¹Institut für Mikrobiologie und Hygiene, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 27, 12203 Berlin, Germany; ²Institut für Biometrie und klinische Epidemiologie, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 20, 12200 Berlin, Germany

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**Objectives:** The aim of this study was to investigate the antiparasitic activity and toxicity of bisphosphonates using a new combined flow cytometry assay.

**Methods:** Using *Toxoplasma gondii* tachyzoites carrying the green-fluorescent protein (GFP), we established a new flow cytometry assay combining testing of *in vitro* and *in vivo* activity plus toxicity of newly synthesized bisphosphonates against *T. gondii*. Toxicity as determined by this assay was compared with toxicity as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

**Results:** In *vivo*, therapeutic efficacy was 100% for bisphosphonates 2F, 3B, 18A, 22A and 30B at 490, 1000, 512, 44.05 and 47.6 μM concentrations, respectively. Toxicity at 100% inhibitory concentrations was 20% for 2F and 3B, 60% for 22A and 30B, and 75% for 18A. In *vivo*, 6 (91A, 203A, 200C, 210A, 204A and 282A) of 15 newly synthesized bisphosphonates (12 nitrogen-containing and 3 n-alkyl) inhibited parasite replication by >50% at a concentration of 100 μM. Whereas substances 91A and 282A (high efficacy) showed moderate and low toxicity (cell viability between 70% and 100%), respectively, toxicities of 203A, 200C, 210A and 204A were 70%, 65%, 80% and 70%, respectively, as determined by flow cytometry. Compounds 290A, 218A, 214A, 266A and 219A inhibited parasite replication by between 20% and 50% at a concentration of 100 μM.

**Conclusions:** Newly synthesized bisphosphonates 2F, 3B, 91A and 282A showed excellent therapeutic activity and low toxicity. These antiparasitic drugs may therefore be promising compounds for use in patients with acute and reactivated toxoplasmosis. The new flow cytometry assay allowed simultaneous determination of therapeutic efficacy and toxicity.

Keywords: infection, treatment, antiparasitic drugs, toxoplasmosis, cell viability

**Introduction**

*Toxoplasma gondii* is a protozoan parasite that infects up to a third of the world’s population.¹ Whereas acute infection usually goes unnoticed, in certain clinical settings (i.e. congenital infection, reactivated infection in immunocompromised patients), parasite replication and the strong inflammatory response result in massive tissue destruction and severe clinical manifestations.¹ The standard therapy of toxoplasmosis is the combination of pyrimethamine plus sulfadiazine or clindamycin.² However, these combinations are often associated with severe side effects including allergic reactions and bone marrow suppression.¹⁻⁵

Bisphosphonates were found to inhibit the growth of intracellular parasites (*T. gondii* and *Leishmania donovani*) *in vitro* and *in vivo*.⁶ Bisphosphonates interfere with the mevanolate pathway, which leads to the synthesis of sterols and polyisoprenoid compounds that are important for parasite survival.⁷ Nitrogen-containing bisphosphonates used for treatment of bone resorption diseases are competitive inhibitors of farnesylpyrophosphate synthase.⁸ The two most active compounds against the replication of *T. gondii* *in vivo* and *in vitro* are n-alkyl-bisphosphonates containing long (*n* = 9 or 10) hydrocarbon chains.⁹ However, toxicity of these compounds has not been investigated thus far. We therefore tested efficacy and...
cytotoxicity of bisphosphonates using flow cytometry for simultaneous analysis of efficacy and toxicity. Compounds 3B, 22A, 30B, 2F and 18A at concentrations between 44 and 1000 μM showed 100% inhibition of parasite replication. In addition, the in vitro activity and cytotoxicity of 15 new bisphosphonates (12 nitrogen-containing and 3 n-alkyl compounds) were tested.

Materials and methods

Antimicrobial agents

Bisphosphonates (Figure 1) for in vitro and in vivo testing were synthesized in the Department of Chemistry, University of Illinois, Urbana Champaign (E. Oldfield).

Parasites and mice

T. gondii RH tachyzoites expressing green-fluorescent protein (GFP) were a gift from Dominique Soldati (Department of Microbiology and Molecular Medicine, University of Geneva, Switzerland). Parasites were maintained in continuous in vitro culture in mouse macrophages (J774A.1).

NMRI mice were maintained under specific-pathogen-free conditions at the Animal Facilities of the Institute for Microbiology and Hygiene, Charité, Berlin, Germany, and were 6–8 weeks old when used.

In vitro testing of bisphosphonates

GFP-RH tachyzoites of T. gondii were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany). Confluent monolayers of

Figure 1. Molecular structures of bisphosphonates.
Flow cytometry assay for testing of antiparasitic drugs against *T. gondii*

Mouse macrophages (2.5 x 10^5/well in 24-well plates) were inoculated with GFP-RH tachyzoites at parasite:cell ratios of 3:1 and 1:2. After incubation at 37°C for 3 h to allow parasite invasion, cultures were rinsed three times with phosphate-buffered saline (PBS) to remove extracellular parasites and incubated with bisphosphonates at concentrations of 50 and 100 μM for 24 and 48 h. Wells were washed with PBS to remove dead cells. Remaining attached cells were detached with cold PBS containing 5% foetal calf serum (FCS), 1% sodium azide (Sigma, Deisenhofen, Germany), harvested and fixed with 4% formaldehyde (Sigma)/PBS for flow cytometry analysis.

In vivo testing of bisphosphonates

NMRI mice were infected intraperitoneally (ip) with 100,000 GFP-expressing RH tachyzoites. Bisphosphonates were dissolved in PBS and were administered ip for 4 days starting at day 1 post-infection. Control mice were infected and treated with drug diluent only. On day 5 post-infection, mice were sacrificed by asphyxiation with CO2 and peritoneal cells were harvested by washing the peritoneal cavity with PBS.

Analysis of in vivo efficacy using flow cytometry

Peritoneal cells were obtained from infected mice that had been treated with different concentrations of drugs or left untreated and examined using flow cytometry. Expression of GFP by *T. gondii* tachyzoites was used to determine the relative percentage of infected cells (FL-1 channel). To counterstain macrophages, a phycoerythrin (PE)-labelled anti-CD86 MAb (Beckton–Dickinson, Mountain View) was used (FL-2 channel); CD86 is a co-stimulatory molecule expressed by macrophages. Cells were visualized on a FACS Calibur™ flow cytometer using CellQuest software (Beckton–Dickinson). Efficacy was determined comparing the relative percentage of infected cells after drug treatment with the relative percentage of infected control cells left untreated. Results are presented as percentage inhibition of infected cells compared with untreated cells.

Combined in vitro analysis of efficacy and toxicity using flow cytometry

Murine macrophages (J774A.1) were used in a new combined analysis of efficacy and toxicity using flow cytometry. Parasite:cell ratios of 3:1 and 1:2 were used. Following incubation with different concentrations of drugs, the efficacy of drugs was determined by calculating the relative percentages of *T. gondii*-infected cells as determined by expression of GFP by the parasite compared with the relative percentages of infected control cells left untreated. The results are presented as percentages of treated versus untreated infected cells. Low numbers of infected cells therefore indicate high efficacy. Percentages of untreated infected control cells were normalized to 100.

Toxicity was determined by calculating total numbers of viable cells (defined as macrophages attached to the bottom of the well) in drug-treated wells compared with the total number of viable cells in untreated control wells. Detached cells were defined as non-viable cells. Results are presented as percentages of viable cells compared with untreated control wells (normalized to 100). Thus, low numbers of live cells indicate high toxicity (≤10%, low toxicity; 11% to 40%, moderate toxicity; ≥40%, high toxicity).

Analysis of cell viability using propidium iodide (PI) and 7-amino-actinomycin D (7-AAD) staining

Mouse macrophages (J774A.1 cell line) were plated in a concentration of 1 x 10^5/well in 96-well plates for 24 h at 37°C. Viability of cells attached to the bottom of the wells and of detached cells in the supernatants was determined by flow cytometry after staining with PI (Sigma) and 7-amino-actinomycin D (BD Biosciences, Heidelberg, Germany). Trypan Blue exclusion was used to determine percentages of viable and non-viable cells by microscopy.

Analysis of cell viability using the MTT test

The cytotoxicity of bisphosphonates on murine macrophages was assessed by the MTT assay as described previously.14 The assay is based on the presence of the enzyme mitochondrial succinate dehydrogenase in live cells; only viable and early apoptotic cells are able to reduce the tetrazolium salt MTT (yellow) resulting in the formation of water-insoluble formazan crystals (blue); dead cells therefore retain the yellow colour of the medium. Briefly, 50 μL of MTT solution (Sigma) was added for 3 h to confluent monolayers of macrophages that had been incubated for 20 h at 37°C with different bisphosphonates at concentrations of 50 and 100 μM (identical to the dosages used to investigate efficacy). Absorbance was measured at 550 nm in an ELISA plate reader (Tecan, Crailsheim, Germany).

Percentage viability was determined by comparison with untreated cells that were considered 100% viable. Cytotoxicity of compounds tested in vivo was analysed in the same manner using concentrations of between 0.049 and 2000 μM (identical to the dosages used in vivo). Results are given as mean viability ± SD pooled from 8 wells/bisphosphonate formulation.

Statistics

To compare differences in percentages of inhibition of *T. gondii* replication by antiparasitic drugs as determined by flow cytometry, the t-test with Bonferroni correction was used. The Kruskal–Wallis approach followed by the Mann–Whitney test was used to compare in vitro toxicity of antiparasitic drugs. A P value of <0.05 was considered significant.

Results

Therapeutic efficacy of bisphosphonates in vivo

Compounds 2F, 3B, 18A, 22A and 30B were tested in vivo against *T. gondii*. Using flow cytometry to determine efficacy, we observed a concentration-dependent inhibition of parasite replication for all compounds (Figure 2a). For each drug, flow cytometry revealed decreasing percentages of GFP-positive cells (upper and lower right quadrant indicating infected cells). Although 100% inhibition of parasite replication was only seen at concentrations of ≥490 for compounds 18A and 2F and at concentrations of ≥1000 μM for compound 3B (Figure 2b). For all drugs, the highest concentration used showed significantly increased efficacy compared with the lowest concentration.
Figure 2. Flow cytometric analysis of the efficacy of bisphosphonates against *T. gondii*. NMRI mice were infected ip with $10^5$ GFP-expressing RH tachyzoites. Starting 1 day, post-infection mice were treated daily with drugs for 4 days at indicated concentrations. On day 5, peritoneal cells were collected and subjected to staining with pre-determined optimal concentrations of PE-labelled CD86-a-mouse antibodies. (a) Flow cytometry plots of GFP staining in untreated and drug-treated mice indicating the percentage of infected cells (upper and lower right-hand quadrants). Due to the high numbers of concentrations of compounds tested, two separate experiments were performed (experiment 1 and experiment 2). (b) Percentage inhibition of *T. gondii* tachyzoite replication by bisphosphonates at different concentrations given in μM. There were three mice in treated groups and four mice in untreated groups. Results are presented as percentage inhibition compared with infected control cells left untreated (giving 0% inhibition, data not shown). NS, not significant. *$P < 0.01$ compared with the concentration giving 100% inhibition ($t$-test with Bonferroni correction).
Toxicity of bisphosphonates

Toxicity of compounds 2F, 3B, 18A, 22A and 30B was determined by the MTT test in murine macrophages. Identical concentrations as those used for testing of therapeutic efficacy were used (Figure 3). Concentrations that showed therapeutic efficacy against *T. gondii* resulted in viabilities ranging from 25% to 80%. The highest toxicity at therapeutic concentrations was observed for compound 18A, whereas compounds 22A and 30B resulted in viabilities of 40%. In these compounds, all concentrations used showed significant toxicity compared with untreated control cells. In contrast, compounds 2F and 3B resulted in viabilities of 80%.

Therapeutic efficacy of new bisphosphonates in vitro

We used the reduction in percentages of infected cells as determined by flow cytometry to analyse therapeutic efficacy of 15 newly synthesized bisphosphonates. First, using a high parasite:cell ratio (3:1), two *n*-alkyl-bisphosphonates (203A and 204A with 12 and 14 hydrocarbon chains, respectively) and four nitrogen-containing bisphosphonates (91A, 200C, 282A and 210A) showed a dose-dependent inhibition of parasite replication of between 50% and 75% (Figure 4a). The *n*-alkyl-bisphosphonate 203A showed 55% inhibition. Nitrogen-containing compounds 91A, 200C, 282A and 210A resulted in between 50% and 65% inhibition. Five additional nitrogen-containing compounds (290A, 218A, 214A, 266A and 219A) showed lower inhibition rates (between 20% and 40%), whereas compounds 227A, 23A, 216A and 261A did not show any therapeutic efficacy against the parasite (data not shown). We then used the more physiological parasite:cell ratio of 1:2 to confirm these results. A similar therapeutic efficacy (as indicated by double arrows, Table 1) that increased with increasing concentration and time of incubation was observed.

Toxicity of new bisphosphonates in vitro

We wished to apply flow cytometry to simultaneously determine toxicity and efficacy assuming that attached cells will be viable whereas detached cells will be non-viable. To confirm this assumption, we compared percentages of viable and non-viable cells attached to the bottom of the well and in the supernatants using staining with PI, 7-AAD and Trypan Blue exclusion (Table 2). We observed that attached cells showed high percentages of viable cells (>84%) using either technique. In contrast, percentages of viable cells in supernatants were 7.7%, 41.6% and 42.3% using PI, 7-AAD and Trypan Blue staining, respectively. Thus, we defined attached cells as viable and detached...
cells as non-viable and investigated the toxicity of new bisphosphonates using flow cytometry determination of live cell percentages (Figure 4b) and the MTT test (Figure 4c). First, using flow cytometry, compounds 203A, 200C, 210A and 204A (which showed the highest therapeutic effect in vitro) showed marked toxicity (percentages of viable cells between 18% and 35%). Only compounds 91A and 282A combined moderate to low toxicity and 50% therapeutic effect in vitro.

Among those compounds which showed 50% in vitro therapeutic efficacy, only 214A resulted in marked toxicity; in contrast, compounds 290A, 218A, 266A and 219A resulted in percentages of viable cells between 70 and 100. Compounds 23A, 216A, 261A and 227A (which showed no in vitro efficacy) displayed percentages of viable cells between 52% and 91%.

Second, the toxicity of the same bisphosphonates was confirmed by the MTT test. The MTT test indicated slight toxicities on murine macrophages for the nitrogen-containing bisphosphonates 91A, 200C, 210A and 204A (which showed parasite inhibition of 50%, 60%, 65% and 70%, respectively). The viability of cells was 90% for all compounds. Interestingly, the n-alkyl-bisphosphonates 204A and 203A that showed parasite inhibition of 75% and 55% inhibition of parasite replication, respectively, displayed the highest toxicity on macrophages (10% and 15% viability, respectively). Nitrogen-containing compounds (290A, 218A, 266A and 219A) that inhibited parasite replication by <50% exerted only moderate toxicity (% viabilities of 85%, 95%, 82% and 87%, respectively). Compounds 227A, 23A, 216A and 261A that did not show therapeutic efficacy against the parasite in vitro exerted moderate toxicity on macrophages (% viability between 61% and 79%).

Comparing toxicity testing by flow cytometry (24 h after incubation) and the MTT test (20 h of incubation), we found agreement in the detection of low toxicity (<50%) for 10 of 15 compounds (91A, 218A, 282A, 219A, 266A, 261A, 290A, 227A, 23A and 216A). Marked toxicity (>50%) was detected by both techniques for 3 of 15 compounds (214A, 203A and 204A). For 2 of 15 compounds, flow cytometry and the MTT test gave contrasting results; in both cases, flow cytometry indicated marked toxicity (>50% for 210A and 200C), whereas low toxicity (<10%) was found in the MTT test. When comparing cytotoxicity as measured by flow cytometry at 24 and 48 h after incubation, all compounds showed increasing toxicity with increasing time of incubation (Table 1).

**Discussion**

Standard treatment of toxoplasmosis is accompanied by severe side effects; thus, alternative therapeutic compounds are urgently needed. The bisphosphonates investigated in this study showed promising therapeutic effects in vitro, with some compounds demonstrating marked toxicity. Further studies are needed to evaluate their potential for in vivo efficacy and to determine any potential side effects.
needed. This study was therefore performed to evaluate the efficacy and toxicity of new bisphosphonates against \textit{T. gondii}. Bisphosphonates have been shown to inhibit replication of \textit{T. gondii} \textit{in vitro} and \textit{in vivo}.\cite{Martin2004,Yardley2004} \textit{in vitro} activity of three nitrogen-containing bisphosphonates [2F (risedronate), 3B and 18A] showing IC\textsubscript{50}s of 0.49 and >200 \(\mu\text{M}\) (not reported for 18A). Yardley \textit{et al.}\cite{Yardley2004} reported that 2F administered for 10 days in dosages of 10 and 20 mg/kg reduced mortality by 40\% and 55\%, respectively. Testing 60 new bisphosphonates \textit{in vitro}, high therapeutic indices were found for \textit{n}-alkyl-bisphosphonates 22A and 30B,\cite{Yardley2004} these compounds reduced mortality of mice following infection with \textit{T. gondii} by 80\% at concentrations of 20 and 10 mg/kg body weight, respectively.

In the current study, we tested the \textit{in vivo} efficacy of 5 bisphosphonates previously reported to show efficacy against \textit{T. gondii} and of 15 newly synthesized bisphosphonates. Since toxicity of bisphosphonates has not been investigated thus far, we established a new combined flow cytometric analysis of efficacy and toxicity. We observed promising \textit{in vivo} activity of nitrogen-containing compound 3B and confirmed the previously reported efficacy of nitrogen-containing compound 2F.\cite{Yardley2004} These
Table 1. Efficacy and toxicity of new bisphosphonates with >50% inhibition of parasite replication using the combined flow cytometry method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>100/100</td>
<td>100/100</td>
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</tr>
<tr>
<td>91A</td>
<td>50</td>
<td>69.39/98.62</td>
<td>44.16/21.87</td>
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<td></td>
<td>100</td>
<td>69.20/95.01</td>
<td>25.98/17.14</td>
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<td></td>
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<td>31.58/64.02</td>
<td>9.31/45.88</td>
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<td>200C</td>
<td>50</td>
<td>90.74/101.55</td>
<td>8.37/29.63</td>
</tr>
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<td></td>
<td>100</td>
<td>34.97/36.27</td>
<td>4.47/41.91</td>
</tr>
<tr>
<td>282A</td>
<td>50</td>
<td>100/118.70</td>
<td>65.08/65.12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100/90.59</td>
<td>43.50/23.84</td>
</tr>
<tr>
<td>210A</td>
<td>50</td>
<td>22.38/70.04</td>
<td>14.01/46.77</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18.48/82.85</td>
<td>5.80/12.44</td>
</tr>
<tr>
<td>204A</td>
<td>50</td>
<td>16.31/37.04</td>
<td>9.88/43.29</td>
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<td></td>
<td>100</td>
<td>32.17/26.98</td>
<td>7.60/39.29</td>
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*aNumbers of viable macrophages following infection with GFP-containing RH tachyzoites of T. gondii (parasite:cell ratio of 1:2) for 24 and 48 h. Following removal of detached (non-viable) cells, cells attached were collected and analysed by flow cytometry.

*bSingle arrows (↓) indicate substances with reduced percentages of live cells indicating toxicity.

*cDouble arrows (↓↓) indicate substances with marked reduction in percentages of infected cells indicating efficacy.

compounds combined high efficacy with low toxicity. In contrast, n-alkyl-containing bisphosphonates 22A and 30B showed high efficacy combined with high toxicity. Therefore, it seems that n-alkyl-bisphosphonates show higher toxicity compared with nitrogen-containing bisphosphonates.

Of the 15 new compounds tested in vitro, we identified four promising compounds (91A, 200C, 282A and 210A) showing >50% inhibition of parasite replication combined with low cytotoxicity measured by MTT assay. Interestingly, all of these were nitrogen containing. n-Alkyl-bisphosphonates 203A and 204A also showed high efficacy but high toxicity. Thus, the higher toxicity of n-alkyl-bisphosphonates observed in our in vitro analysis was confirmed by in vitro studies. Our results therefore underline that testing of efficacy of new drugs should always be combined with toxicity testing.

To simultaneously determine efficacy and toxicity, we established a new combined technique using flow cytometry. To determine efficacy, we compared the percentages of infected cells in drug-treated wells with the percentages of infected cells in untreated control wells using parasites expressing the GFP easily detected in the fluorescein channel (FL-1). Assuming that attached cells are viable and cells in the supernatant are non-viable, we determined toxicity in the identical wells in parallel by counting the total number of attached (viable) cells following removal of non-viable cells in the supernatants. We further compared this approach with results obtained by MTT testing. In 10 of 15 compounds (91A, 218A, 282A, 219A, 266A, 261A, 290A, 227A, 23A and 216A), flow cytometry confirmed the low toxicity results of MTT testing and also confirmed the marked toxicity of 3 of 15 compounds (214A, 203A and 204A). For two compounds (210A and 200C), flow cytometry indicated higher toxicities compared with the MTT test. These results suggest that our new approach using flow cytometry is more sensitive than the MTT test to detect cytotoxicity. This may be caused by the fact that our flow cytometry technique appears to detect a significant number of viable cells in the supernatants thus giving ‘false-positive’ results for toxicity (Table 2).

As described, the new combined flow cytometry technique can only be used for in vitro analysis. However, adding a dye specific for the distinction between viable and non-viable cells, e.g. propidium iodide, ex vivo applications are feasible.

In the past, a variety of techniques have been used to test efficacy of drugs against T. gondii.15–17 Initially, testing of antiparasitic efficacy was performed by microscopy following staining of parasites18 which is hampered by low accuracy. Incorporation of [3H]uracil as a measurement to detect loss of parasite replication is more accurate but requires a laboratory equipped to handle radioactivity.19,20 β-Galactosidase-expressing parasites and their detection by chlorophenol red-β-N-galactopyranoside-based colorimetric assays have also been applied.21 In vivo, the antiparasitic activity of drugs has been evaluated by microscopic determination of parasite numbers and/or mortality.22 More recently, PCR has been applied to determine numbers of parasites following treatment of mice.23,24 Flow cytometry has been used in the past to investigate the efficacy of drugs against Leishmania infantum and Giardia lamblia.25,26 The efficacy of drugs against L. infantum was determined by measuring non-protein thiol using mercury orange and total proteins using FITC.26 Viability of trophozoites of G. lamblia was analysed by

Table 2. Percentages of viable and non-viable macrophages as determined by staining with PI, 7-AAD and Trypan Blue exclusion

<table>
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<td></td>
<td>viable</td>
<td>non-viable</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>94.50 ± 2.41</td>
<td>5.52 ± 6.14</td>
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<td>7-AAD</td>
<td>86.61 ± 6.26</td>
<td>13.39 ± 6.26</td>
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<tr>
<td>Trypan Blue</td>
<td>84.25 ± 3.43</td>
<td>15.76 ± 3.43</td>
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<tbody>
<tr>
<td></td>
<td>viable</td>
<td>non-viable</td>
<td></td>
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<tr>
<td>PI</td>
<td>7.75 ± 3.33</td>
<td>92.26 ± 3.33</td>
<td></td>
</tr>
<tr>
<td>7-AAD</td>
<td>41.63 ± 11.05</td>
<td>58.38 ± 11.05</td>
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</tr>
<tr>
<td>Trypan Blue</td>
<td>42.33 ± 3.30</td>
<td>57.67 ± 3.30</td>
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Cells were plated at a concentration of 1 × 10⁵/well; supernatants were obtained after 24 h. Viability of cells was determined by flow cytometry (PI and 7-AAD) or Trypan Blue exclusion as described in the Materials and methods section. Numbers indicate mean percentages of cells ± SD.
Flow cytometry assay for testing of antiparasitic drugs against T. gondii

staining with fluorescence diacetate and propidium iodide for live and dead trophozoites, respectively.\(^3\) Recently, efficacy of antibacterial agents against several Mycoplasma species was investigated in vitro by staining Mycoplasma with SYBR green for nucleic acids; propidium iodide was used to stain nucleic acids in cells with damaged membranes.\(^2\) These flow cytometric techniques were exclusively used for in vitro investigation of drug activities; however, none of these studies has attempted to simultaneously determine the efficacy and toxicity of drugs. Thus, flow cytometry as described in the present study harbours the advantage of a combined analysis of efficacy and toxicity thereby reducing the number of samples by 50%. However, it requires microorganisms genetically altered to carry a fluorescent protein and a flow cytometer.

In conclusion, the present study revealed high efficacy and low toxicity of new nitrogen-containing bisphosphonates (91A and 282A). The new flow-cytometry-based technique combining simultaneous measurement of efficacy and toxicity should prove valuable to test the efficacy of new anti-infective drugs.

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Transparency declarations

None to declare.

References

17. Ferreira RA, Oliveira AB, Ribeiro MF et al. Toxoplasma gondii: in vitro and in vivo activities of the hydroxynaphthoquinone 2-hydroxy-3(1'-propen-3-phenyl)-1,4-naphthoquinone alone or combined with sulfadiazine. Exp Parasitol 2006; 113: 125–9.
