



RESEARCH PAPER



## Nanoemulsions of sulfonamide carbonic anhydrase inhibitors strongly inhibit the growth of *Trypanosoma cruzi*

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### ABSTRACT

Sulfonamide carbonic anhydrase (CA, EC 4.2.1.1) inhibitors targeting the  $\alpha$ -class enzyme from the protozoan pathogen *Trypanosoma cruzi*, responsible of Chagas disease, were recently reported. Although many such derivatives showed low nanomolar activity *in vitro*, they were inefficient anti-*T. cruzi* agents *in vivo*. Here, we show that by formulating such sulfonamides as nanoemulsions in clove (*Eugenia caryophyllus*) oil, highly efficient anti-protozoan effects are observed against two different strains of *T. cruzi*. These effects are probably due to an enhanced permeation of the enzyme inhibitor through the nanoemulsion formulation, interfering in this way with the life cycle of the pathogen either by inhibiting pH regulation or carbonylating reactions in which bicarbonate/CO<sub>2</sub> are involved. This type of formulation of sulfonamides with *T. cruzi* CA inhibitory effects may lead to novel therapeutic approaches against this orphan disease.

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## 1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is endemic in regions of Central and South America. In Latin America, five to eight million people are infected with this protozoan<sup>1</sup>. Infection starts by blood-sucking triatomine bugs, but others transmission routes are possible, such as organ transplantation, congenital contamination, blood transfusion as well as contaminated foods and drinks<sup>2,3</sup>. Due to these modes of transmission the disease is spreading to nonendemic countries including Australia, Canada, Japan, Spain, and the USA<sup>2,3</sup>. The drugs used for the treatment of the disease are the nitroheterocyclic compounds benznidazole and nifurtimox but both of them induce severe side effects and cross-resistance<sup>4</sup>. New therapeutic approaches and new drugs are investigated constantly, and in the present work, nanoemulsions (NEs) of sulfonamides derivatives with inhibitory effects against the carbonic anhydrase (CA, EC 4.2.1.1) from *Trypanosoma cruzi* were developed.

NEs have been widely used in the pharmaceutical area as drug carriers. Due to the existence of polar and apolar phases at the interfacial domain, NEs are versatile release systems able to encapsulate drugs with variable solubility<sup>5</sup>. The majority of NEs are dispersions of oil droplets in water with diameter between 20 and 200 nm. NEs present small droplet size that allows the Brownian motion of the drops retarding their sedimentation or coalescence. Thus, NEs present kinetic stability<sup>6</sup>, promoting tissue permeation and penetration of drugs. Their nanometric droplets have large relative surface area, facilitating the contact of the nanocarrier

with the biological membrane or tissue, and consequently favouring drug permeation and retention. The surfactants included in the NEs can promote reduction of the surface tension between the droplets and biological tissue or membrane, improving the drug's spreadability and bioadhesion. These droplets can also act as a reservoir system for sustained drug release. Moreover, the main advantages of nanocarriers are the ease of preparation, possibility of industrial-scale production, and high thermodynamic stability<sup>7</sup>.

NE preparations have been used to improve drug activity. 2-(Butylamino)-1-phenyl-1-ethanethiosulfuric acid (BphEA) is a promising schistosomicidal drug; however, it presents low solubility in water and low effectiveness against the parasite. The NEs containing BphEA were produced utilizing ultrasound, oil phase with medium-chain triglycerides (coconut oil) and stearylamine, and mixtures of nonionic surfactants (Span 80 and Tween 80). The drug in NE form presented more schistosomicidal activity than the solution. The NE interacted with the surface membrane of the parasite promoting the permeation of the drug and schistosomicidal activity<sup>8</sup>. Zinc phthalocyanine (ZnPc) and chloroaluminum phthalocyanines (ClAlPc) are photosensitizers used in photodynamic therapy of cancer. However, these photosensitizers present water solubility problems. NEs were used to solve the solubility problems leading to enhanced effectiveness of these photosensitizers. The NEs containing ZnPc or ClAlPc were produced utilizing ultrasonic processor, oil clove, and nonionic surfactants (Lutrol<sup>®</sup> F-68). The results showed that the photosensitizers in the NE form were more affective in the elimination of tumour

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cells (cells A549, human lung carcinoma cells) than the photosensitizer solution alone<sup>9</sup>.

Carbonic anhydrase (CA, EC 4.2.1.1) inhibition has pharmacologic applications in various fields, with antiglaucoma<sup>10</sup>, diuretics<sup>11</sup>, antiepileptics<sup>12</sup>, antiobesity<sup>13</sup>, and antitumour agents<sup>14</sup> belonging to various classes of such pharmacological agents (sulfonamides, coumarins, dithiocarbamates, etc.)<sup>10–14</sup>. Recently, the potential use of CA inhibitors (CAIs) as anti-infectives also started to be considered, with antibacterials<sup>15–17</sup>, antifungals<sup>18,19</sup>, and antiprotozoan agents<sup>20–22</sup>, being investigated in the search for agents devoid of the resistance problems common to most classes of clinically used such agents<sup>23</sup>. We have, for example, reported that *T. cruzi*, the etiological agent of Chagas diseases<sup>20</sup> encodes for an  $\alpha$ -CA, called TcCA<sup>20</sup>. This enzyme was inhibited *in vitro* by many sulfonamides in the low nanomolar or subnanomolar range<sup>24,25</sup>. However, *in vivo*, the growth of the parasite was not inhibited by such sulfonamides<sup>24,25</sup>. Only some heterocyclic thiols<sup>20</sup> and hydroxamates<sup>26,27</sup> did show *in vivo* efficacy as anti-*T. cruzi* agents (and they also acted as efficient *in vitro* TcCA inhibitors)<sup>24–28</sup>, and we considered that this might be due to the lack of permeability of the sulfonamides through the biological membranes of the protozoan. This is the reason why we decided to investigate the formulation of such sulfonamides, highly effective as TcCA inhibitors in NEs, in order to enhance their bioavailability and penetrability through membranes. Here, we report that sulfonamide TcCA inhibitors formulated as NEs in clove oil, potentially inhibit the growth of *T. cruzi ex vivo*, showing thus a potential as a novel class of antitrypanosomal drugs.

## 2. Materials and methods

### 2.1 Chemistry

Sulfonamides **3F**, **3G**, **3W**, **5B**, **5C**, and **5D** used in the experiments were reported in an earlier work from our groups<sup>21</sup>.

### 2.2 Materials

Clove oil (*Eugenia caryophyllus*) was purchased from Ferquima Ltd. (Brazil). Pluronic F-127, a nonionic block-copolymer surfactant of (poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)) (EO<sub>100</sub>PO<sub>66</sub>EO<sub>100</sub>), with MW 12,600, and HLB 22, was

purchased from Sigma Aldrich (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM), resazurin, Benzimidazol (Bz): 2-nitro-imidazole-(N-benzil-2-nitro-1imidazoleacetamide), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from LGC Biotecnologia (São José, Cotia, Brazil).

### 2.3 NE preparation

The oil-in-water (O/W) NEs were prepared by high-energy method (Figure 1) using an ultrasound processor (Hielscher model UP100H), according to a method adapted from literature<sup>29</sup>. Oil phase was prepared by sulfonamides dissolution in the clove oil. A 5 mg of drug was weighted in an eppendorf and 1 ml of clove oil was added. The tube was agitated for 1 min for obtaining of the drug solution (5 mg/ml). Aqueous phase were prepared by adding 1 g of Pluronic F127<sup>®</sup> in 8 mg of water. Then 1 ml of oil phase (drug dissolved in clove oil) was added to 9 ml of aqueous phase (Pluronic F127 in water) under constant ultrasound homogenization (amplitude 80%, continuous cycle n. 1) during 5 min in an ice bath at 5 °C to prevent heating of the dispersion. A transparent NE was obtained at a concentration of 500 µg/ml (Figure 1).

### 2.4 Determination of droplet size

Determination of droplet size and polydispersity index (PDI) were measured using the dynamic light scattering (DLS) method with a Malvern model 905 NanoSizer<sup>®</sup> (UK). NEs were diluted in distilled water at 1:10 and analyzed in a cell with 1 cm optical path at room temperature (25 °C). These analyses were conducted in three runs with fifteen readings. The values shown are the mean ± standard deviation of three measurements for each formulation. The PDI reflects the sample quality in the parameter homogeneity of the droplet diameter. PDI results lower than 0.3 were considered satisfactory<sup>30</sup>.

### 2.5 *T. cruzi* parasites

Epimastigote forms of the *T. cruzi* clone Dm28c (lineage TCII)<sup>31</sup> and Y(lineage TCI)<sup>32,33</sup> strains obtained from the Laboratory of

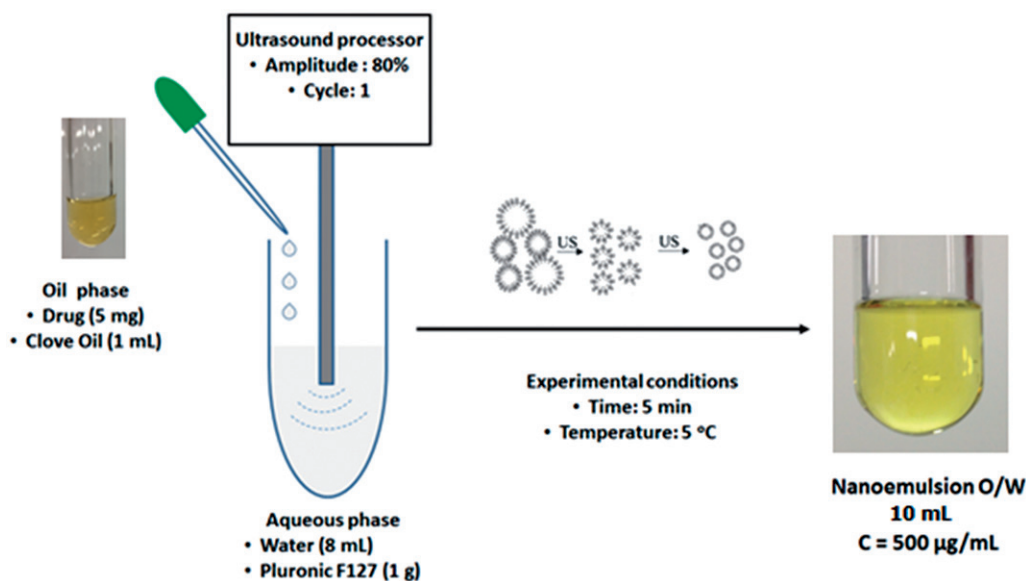


Figure 1. Preparation of nanoemulsion by high-energy method.

Cellular Ultrastructure (both laboratories of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) were used. The parasites were maintained in PBHIL medium supplemented with 10% bovine serum (FBS) at 28 °C<sup>34</sup>.

## 2.6 RAW 264.7 macrophage cell line culture

RAW 264.7 macrophages were obtained from the National Institute of Metrology, Quality and Technology (Instituto Nacional de Metrologia, Qualidade e Tecnologia, INMETRO) and maintained in DMEM medium supplemented with 10% FBS at 37 °C in a 5% controlled CO<sub>2</sub> atmosphere. Cell maintenance was performed every 48–72 h, time necessary for cells to achieve confluent monolayers.

## 2.7 Evaluation of NEs activity on *T. cruzi* epimastigotes

The evaluation of NEs antiprotozoal activity was performed by successive microdilutions in 96 well plates ( $1.8 \times 10^6$  parasites/well) NEs in the PHBIL medium supplemented with 10% FBS in the following concentrations: 128, 64, 32, 16, 8, 4, 2, and 1 μM. The experiment controls were: negative control (culture medium with parasite) and positive culture (culture medium with parasite), and Benznidazole (as reference drug) was also progressively diluted with the parasite. The Minimum inhibitory concentration (MIC) for epimastigotes was performed with resazurin as an indicator of cellular metabolic function and it was determined as the lowest concentration capable of inhibiting *in vitro* growth of the parasites<sup>35</sup>. The determination of IC<sub>50</sub> and IC<sub>90</sub> (concentration of drug that reduces epimastigotes proliferation by 50%) was obtained by distance from the line from the inhibition values (%).

## 2.8 Cytotoxicity essay in macrophages

Sulfonamide NEs cytotoxicity was performed using tetrazolium dye (MTT) colorimetric assay. RAW 264.7 macrophages were harvest after confluent monolayer achievement. The cells were washed twice with PBS and a cellular suspension of 10<sup>6</sup> cells/ml was prepared in fresh DMEM culture medium. Aliquots of 100 μl of the

cellular suspension were placed into polystyrene 96-well plates, and then incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 6 h to allow for adherence of macrophages. After this period, the adherent cells were subjected to treatment with several concentrations of the sulfonamide NEs (1–128 μM), and then incubated for additional 48 h. Finally, 20 μl of a MTT solution (5 mg/ml) were added to each well and the plates incubated for 4 h<sup>36</sup>. Macrophage viability was determined after formazan crystals solubilization with DMSO followed by the absorbance measurement at 570 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA).

## 2.9 Determination of selectivity index

A selectivity index (SI) was calculated and is defined as the RAW IC<sub>50</sub> value divided by the *T. cruzi* IC<sub>50</sub> value ( $SI = CC_{50}/IC_{50}$ ), which expresses the safety index of the tested substance<sup>37</sup>. Benznidazole (Sigma-Aldrich, Milan, Italy) was kept as a positive control drug for the cytotoxicity assay on RAW 264.7.

## 2.10 Flow cytometry

The analysis of the externalization of phosphatidylserine was performed using annexin V fluoresceine isothiocyanate (AV FITC) and propidium iodide, PI (Santa Cruz Biotechnonology, Santa Cruz, CA). The cells were analysed by flow cytometry (FACS Calibur-Beckton-Dickinson), 50,000 events were analysed by the Paint-a-gate program and values were expressed as percentage of cells positive for a given marker relative to the total number of cells: FITC-labelled cells (viable cells), PI-labelled cells (apoptotic cells), and cells with ruptured cell membrane (necrotic cells will be double-labelled).

## 2.11 Statistical analysis

The data of the experiments are being carried out through the program Prism 5.01 GraphPad (GraphPad Software, Los Angeles, CA), being considered values statistically significant those with values  $p < 0.05$ . The values were expressed as the mean ± standard

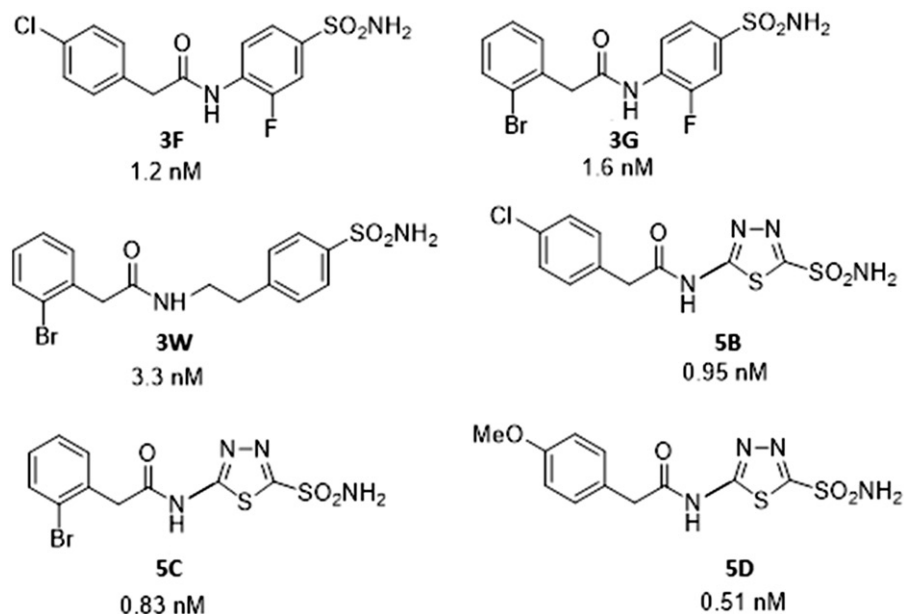


Figure 2. Sulfonamides 3F, 3G, 3W, 5B, 5C, and 5D used in the study and their TcCA inhibitory action.

deviation (SD). The analysis of variance was done by the Student–Newman–Keuls test for comparison between means.

### 3. Results and discussion

#### 3.1 Preparation of sulfonamide NEs

Drugs **3F**, **3G**, **3W**, **5B**, **5C**, and **5D** act as highly efficient TcCA inhibitors *in vitro*<sup>25</sup>, with inhibition constants ranging between 0.51 and 3.3 nM (Figure 2). However, as mentioned above, they did not show *in vivo* anti-trypanosomal effects<sup>25</sup>. This is the reason why they were formulated as NEs in clove oil.

Sulfonamides **3F**, **3G**, **3W**, **5B**, **5C**, and **5D** were dissolved efficiently in clove oil in the concentration of 5 mg/ml. The NEs were produced with 10% of oil phase. NEs were prepared also without

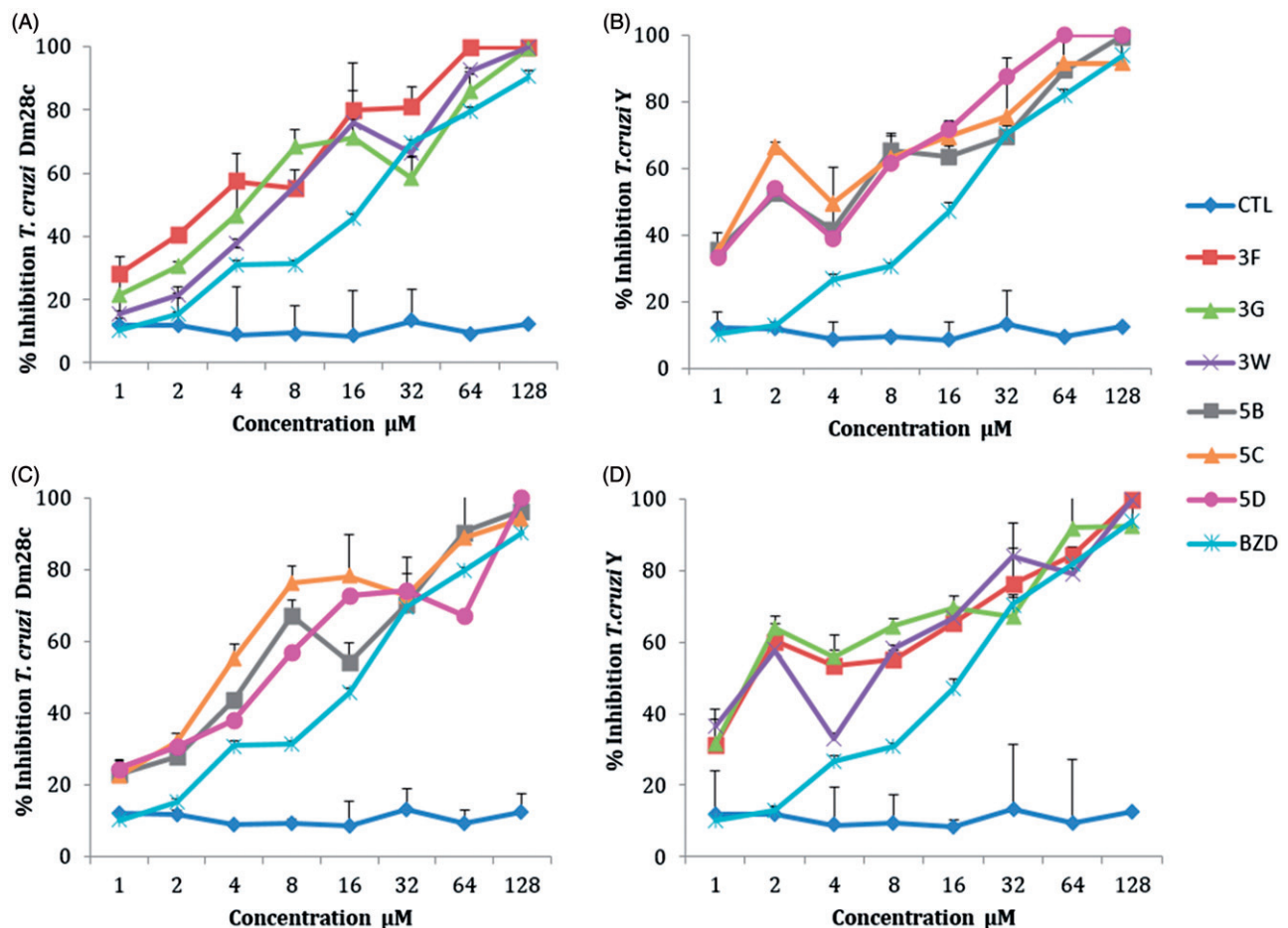
the drug, in order to evaluate the stability, droplet size, and PDI. The NEs obtained were yellow and transparent suggesting that the system was homogeneous with small droplet size (Table 1). As phase separation and precipitation of the drug were not observed, the NEs were considered stable in the concentration of 500 µg/ml.

NEs without the drug presented an average size of 31.54 nm. The NEs containing drug presented average sizes between 35 and 100 nm, depending on the drug. The lowest average size was exhibited by NE **5D** with 35.09 nm. NE-**3G** and NE-**3W** exhibited the larger average size values with 100.63 and 97.34 nm, respectively. The NEs presented PDI below 0.3, indicating that the size distribution is homogeneous and monomodal. Thus, we conclude that the inclusion method of the drugs in NEs was adequate producing nanostructured samples with drops below 100 nm and size distribution homogeneous and monomodal.

**Table 1.** NEs size and polydispersity index

Formulation/drug	Drug (mg)	Oil Clove (ml)	AP (ml)	Size (nm)	PDI	Stability
NE	–	1	9	31.54 ± 0.413	0.105 ± 0.012	Stable
NE-3F	5	1	9	60.12 ± 2.36	0.274 ± 0.033	Stable
NE-3G	5	1	9	100.63 ± 2.05	0.262 ± 0.008	Stable
NE-3W	5	1	9	97.34 ± 2.82	0.264 ± 0.15	Stable
NE-5B	5	1	9	44.83 ± 0.753	0.123 ± 0.078	Stable
NE-5C	5	1	9	53.99 ± 1.12	0.233 ± 0.003	Stable
NE-5D	5	1	9	35.09 ± 0.575	0.165 ± 0.019	Stable

AP: aqueous phase containing tensioactive (Pluronic F127) and water, drug concentration 500 µg/mL, mean ± SD (from three different determinations).



**Figure 3.** Inhibition effects of different concentrations of nanoemulsions with sulfonamide derivatives (1–128 µM) **3F**, **3G**, **3W**, **5B**, **5C**, **5D**. (A and C) Epimastigotes *T. cruzi* Dm28c strain; (B and D) epimastigotes *T. cruzi* Y strain, after 5 days of incubation. CTL control nanoemulsions without sulfonamide derivatives, BZD: benznidazole reference drug (1–128 µM).



### 3.2 Anti-*T. cruzi* activity in vivo

The percentage inhibition of epimastigote forms at different concentrations of NEs are shown in Figure 3. The sulfonamide **3F** in a concentration of 4  $\mu\text{M}$  inhibited 57% and 43.51% the epimastigotes forms of DM28c and Y strain of *T. cruzi*, respectively. The **5C** derivative showed a significant inhibition of 55.2% (for DM28c strain) and of 49.4% (Y strain) at 4  $\mu\text{M}$  concentration of drug.

The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) values of the sulfonamides NEs were lower than the benznidazol (20.63  $\mu\text{M}$ ) for the epimastigote forms of both strains of *T. cruzi*. (Y and DM28c).

**Table 2.**  $\text{IC}_{50}$  and  $\text{IC}_{90}$  values derived from growth inhibition assays of *Trypanosoma cruzi* (DM28c, Y) and determination of cytotoxicity ( $\text{CC}_{50}$ ), selectivity index ( $\text{SI}_{50}$ ) of **3F**, **3G**, **3W**, **5B**, **5C**, **5D** NEs.

	Drug nanoemulsions						
	3F	3G	3W	5B	5C	5D	BZN
Tc DM28c							
$\text{IC}_{50}^1$ $\mu\text{M}$	3.54 <sup>a</sup> $\pm$ 1.53	5.66 <sup>b</sup> $\pm$ 1.62	7.36 <sup>b</sup> $\pm$ 1.54	6.24 <sup>b</sup> $\pm$ 0.18	3.98 <sup>a</sup> $\pm$ 0.24	6.69 <sup>b</sup> $\pm$ 1.85	20.63 <sup>c</sup> $\pm$ 1.831
$\text{IC}_{90}^2$ $\mu\text{M}$	49.56 <sup>a</sup> $\pm$ 9.61	84.87 <sup>b</sup> $\pm$ 5.16	68.64 <sup>c</sup> $\pm$ 23.47	84.46 <sup>b</sup> $\pm$ 6.80	64.34 <sup>c</sup> $\pm$ 6.47	120.54 <sup>d</sup> $\pm$ 7.89	>128
$\text{CC}_{50}^3$ $\mu\text{M}$	8.13 <sup>a</sup> $\pm$ 1.19	6.77 <sup>b</sup> $\pm$ 1.07	3.21 <sup>c</sup> $\pm$ 0.55	6.51 <sup>b</sup> $\pm$ 1.11	8.04 <sup>a</sup> $\pm$ 1.33	6.75 <sup>b</sup> $\pm$ 0.98	127.54 <sup>d</sup> $\pm$ 12.04
$\text{SI}_{50}^4$	2.25 <sup>a</sup> $\pm$ 0.17	1.20 <sup>b</sup> $\pm$ 0.21	0.44 <sup>c</sup> $\pm$ 0.12	1.08 <sup>b</sup> $\pm$ 0.11	2.02 <sup>a</sup> $\pm$ 0.22	1.05 <sup>b</sup> $\pm$ 0.09	5.54 <sup>d</sup> $\pm$ 1.82
TcY							
$\text{IC}_{50}^1$ $\mu\text{M}$	2.83 <sup>a</sup> $\pm$ 0.71	2.27 <sup>a</sup> $\pm$ 0.56	3.51 <sup>b</sup> $\pm$ 0.12	3.47 <sup>b</sup> $\pm$ 0.35	2.15 <sup>a</sup> $\pm$ 0.29	3.27 <sup>b</sup> $\pm$ 0.53	21.92 <sup>c</sup> $\pm$ 1.67
$\text{IC}_{90}^2$ $\mu\text{M}$	>128	83.61 <sup>a</sup> $\pm$ 38.25	114.97 <sup>b</sup> $\pm$ 8.16	82.03 <sup>a</sup> $\pm$ 8.01	78.04 <sup>a</sup> $\pm$ 32.46	52.67 <sup>c</sup> $\pm$ 7.70	>128
$\text{CC}_{50}^3$ $\mu\text{M}$	8.13 <sup>a</sup> $\pm$ 1.19	6.77 <sup>b</sup> $\pm$ 1.07	3.21 <sup>c</sup> $\pm$ 0.55	6.51 <sup>b</sup> $\pm$ 1.11	8.04 <sup>a</sup> $\pm$ 1.33	6.75 <sup>b</sup> $\pm$ 0.98	127.54 <sup>d</sup> $\pm$ 12.04
$\text{SI}_{50}^4$	2.89 <sup>a</sup> $\pm$ 1.02	3.09 <sup>a</sup> $\pm$ 1.33	0.44 <sup>b</sup> $\pm$ 0.06	1.95 <sup>c</sup> $\pm$ 0.97	5.09 <sup>d</sup> $\pm$ 1.41	1.76 <sup>c</sup> $\pm$ 0.30	5.89 <sup>d</sup> $\pm$ 0.81

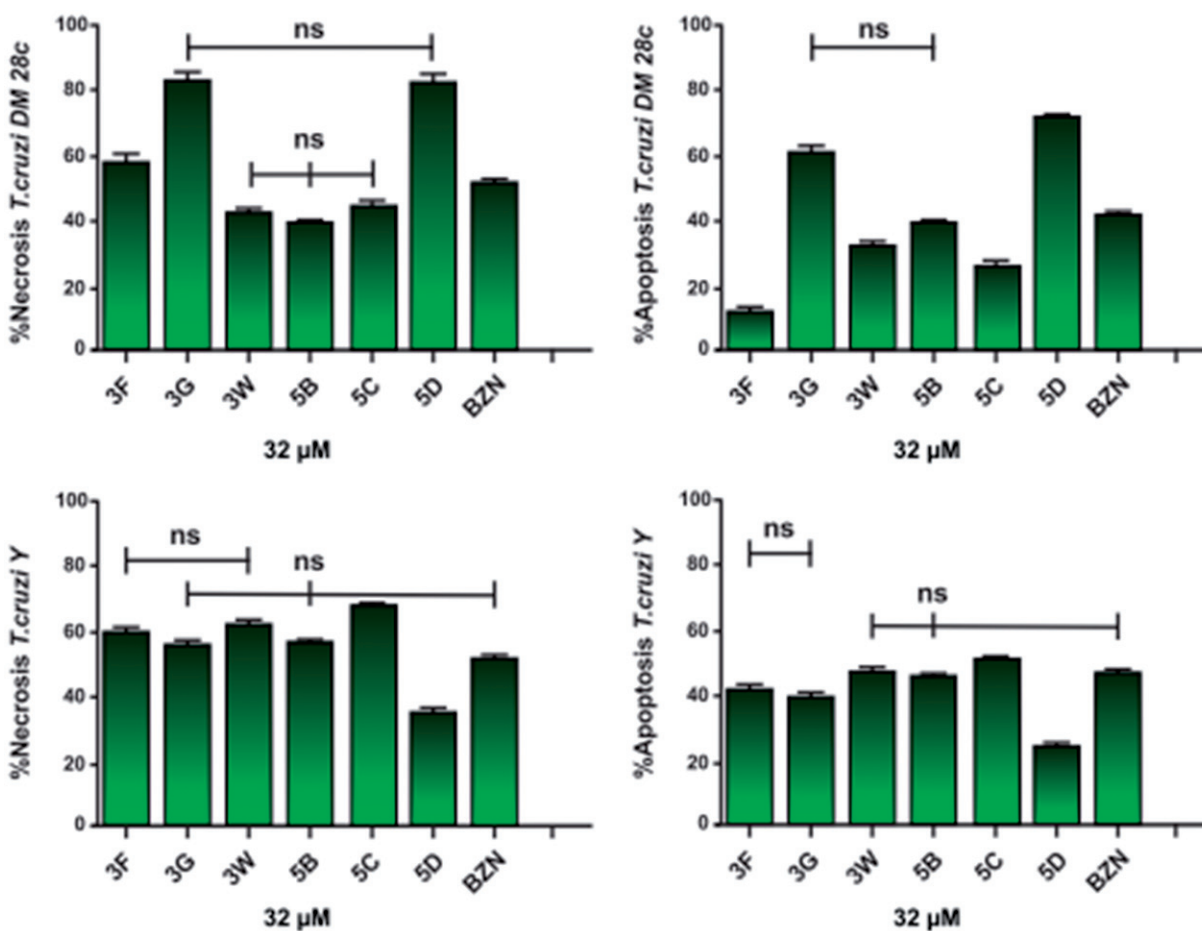
<sup>1</sup>Concentration in  $\mu\text{M}$  which reduced the proliferation of epimastigotes by 50%.

<sup>2</sup>Concentration in  $\mu\text{M}$  which reduced the proliferation of epimastigotes by 90%.

<sup>3</sup>Concentration in cytotoxic  $\mu\text{g ml}^{-1}$  to 50% of RAW 267.4 cells.

<sup>4</sup> $\text{SI}_{50}$  Selectivity index of 50% =  $\text{CC}_{50}/\text{IC}_{50}$ .

a,b,c,d; in the rows, means followed by different letters differ statistically ( $p < .05$ ).



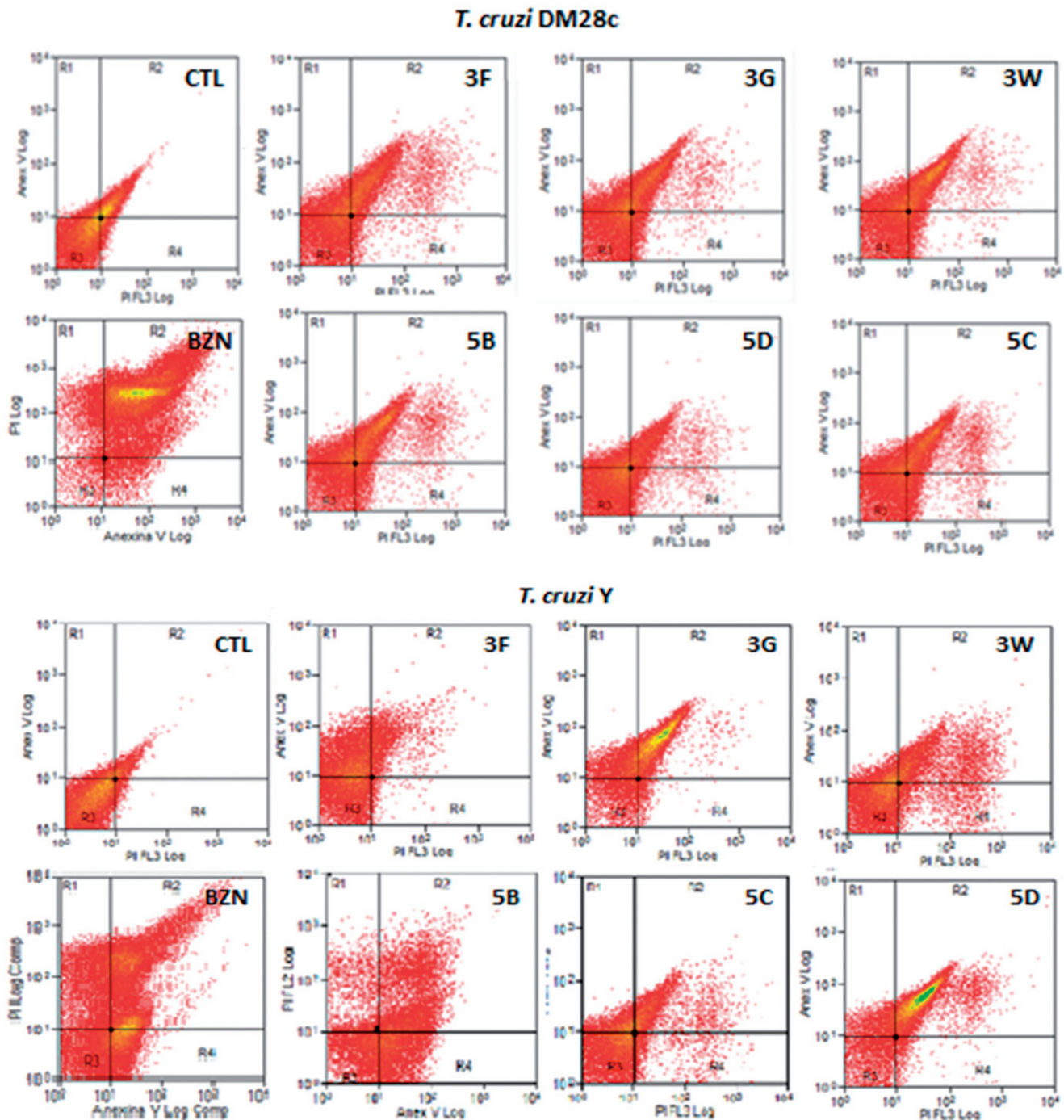
**Figure 4.** Representative graphs of flow cytometric analysis for nanoemulsions of the sulfonamides **3F**, **3G**, **3W**, **5B**, **5C**, **5D**, and benznidazole (BZN) at 32  $\mu\text{M}$ . (A, C) Necrosis using propidium iodide (PI) and (B, D) apoptosis using annexin V-FITC (ANV).

which is comparable to that of the standard drug benznidazol (Table 2).

### 3.3 Flow cytometry

Apoptosis and necrosis are different types of cell death. Apoptosis, or programmed cell death, is a form of cell death that is generally triggered by normal physiological processes. On the other hand, necrosis is a premature cell death that can be caused by external factors. They can be differentiated by flow cytometry using distinct dyes. Annexin V (AV) is a marker of apoptosis, being a  $Ca^{2+}$ -dependent phospholipid-binding protein with a high affinity for

phosphatidylserine. Propidium iodide (PI) is a fluorescent dye necrosis indicator. It is a cell-impermeant dye that intercalates DNA and RNA of cells with damaged plasma membrane. The NEs containing the sulfonamides **3G**, **5D**, and **3F** lead the cell death by necrosis, in the following proportions, of 82.41%, 81.26% and 57.03%, respectively for the *T. cruzi* Dm28c strain, being more effective than the reference drug benznidazole (effect of 51.16%). The drugs **3G**, **5B**, and **5D** induced more apoptosis than benznidazole too. Similar values were found for the *T. cruzi* Y strain. The sulfonamide NEs killed the parasites by necrosis in the proportion of 54.80% for **3G**, of 62.43% for **3W**, of 55.67% for **5B**, and of 67.01% for **5C**. These results indicate the sulfonamides in glove oil



**Figure 5.** Histogram of epimastigotes representative of apoptosis analysis by flow cytometry using propidium iodide (PI) and annexin V-FITC. CTL: control without sulfonamides derivatives (A) *T. cruzi* Dm28c and (B) *T. cruzi* Y Nanoemulsions of **3F**, **3G**, **3W**, **5B**, **5C**, **5D** sulfonamides derivatives at 32  $\mu$ M. R1: Cells marked with PI only (necrosis); R2: cells labelled with annexin V and PI (late apoptosis); R3: unlabelled cells (viable cells); R4: cells marked only with annexin V (early apoptosis).

NEs were more effective in their anti *T. cruzi* effects than benznidazole. Apoptosis occurred only with **5C** in a statistically significant manner (Figure 4). In addition it was observed that most AV-positive cells were also positive for PI, suggesting that apoptotic cells evolved to secondary necrosis, with the possibility that annexin is bound to internal phosphatidylserine residues after the membrane integrity was lost (Figures 4 and 5).

#### 4. Conclusions

Sulfonamide CAIs have various pharmacologic applications, as shown in the introduction of this paper. Although we have discovered low nanomolar *in vitro* TcCA inhibitors, in our first reports we could not evidence *in vivo* efficacy of such agents in interfering with the life cycle of the pathogen<sup>20,22</sup>. Thus, we have hypothesized that this lack of effect is due to problems of permeability of the sulfonamide through the biological membrane of the protozoan. This is the reason why we have explored the formulation of these CAIs as NEs in clove oil. The approach was in fact successful, since several sulfonamide strong TcCA inhibitors indeed showed significant anti-*T. cruzi* effects, against two different strains of the pathogen. These effects are probably due to an enhanced permeation of the enzyme inhibitor through the NE formulation, interfering in this way with the life cycle of the pathogen, either by inhibiting pH regulation or carboxylating reactions in which bicarbonate/CO<sub>2</sub> are involved. This type of formulation of sulfonamides with *T. cruzi* CA inhibitory effects may lead to novel therapeutic approaches against this orphan disease.

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#### Disclosure statement

The authors do not declare any conflict of interest.

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