In vitro genotoxicity of nitroimidazoles as a tool in the search of new trypanocidal agents

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BACKGROUND Only benznidazole (Bnz) (1) and nifurtimox (Nfx) (2) are licensed for the treatment of Chagas disease although their safety and efficacy profile are far from ideal. Farmanguinhos from Fiocruz has developed seven nitroimidazole compounds (4-10) analogs of megazol (3).

OBJECTIVES To evaluate whether the genotoxic effect of 3 was abolished in the seven nitroimidazoles (4-10) analogs using the in vitro alkaline comet assay (CA) and the in vitro cytogenesis-block micronucleus assay (CBMN) in whole human blood cells (WHBC) and correlate this effect with their trypanocidal activity using bloodstream trypomastigote forms of Trypanosoma cruzi.

METHODS The toxicity of 3-10 to WHBC in the in vitro CA was determined using the fluorescein diacetate/ethidium bromide assay. DNA damage in the in vitro CA was evaluated according to tail size in four classes (0-3) and methyl methane-sulfonate (MMS) was used as a positive control. The cytotoxicity of 3-10 to WHBC in the CBMN was measured using the cytokinesis-block proliferation index and the replication index. The number of the micronucleate cells in 2,000 binucleate cells by experimental group was determined. Mitomycin C and N-deacetyl-N-methylcolchicine were used as positive controls.

FINDINGS Compound 3 showed a significant DNA strand break effect through the in vitro CA and highly significant clastogenic and/or aneugenic effect in the CBMN. Compounds 5, 6, 8, 9 and 10 showed negative results in the CBMN and positive results in the in vitro CA, while the inverse effect was observed for 4 and 7.

MAIN CONCLUSIONS Compound 10 was the most promising to proceed with the development as a drug candidate in the treatment of Chagas disease showing absence of chromosomal cytogenetic damage and high activity against T. cruzi, about two times higher than 3 and the clinical drug 1.

Key words: genotoxicity - mutagenicity - megazol - nitroimidazoles

Chagas disease caused by the protozoan Trypanosoma cruzi remains a major social and public health problem in Latin America and is regarded as a neglected tropical disease by World Health Organization (WHO). WHO estimates 5-7 million people are infected with T. cruzi worldwide, mainly in Latin America highlighting Argentina, Brazil, Mexico and Bolivia. In the last two decades, cases have been found in European countries, Japan, Australia and the USA, resulting from the immigration of infected individuals.

Only two drugs, the 2-nitromidazole benznidazole (Bnz) (1) and the 5-nitrofuran nifurtimox (Nfx) (2) (Fig. 1), are licensed for the treatment of Chagas disease, although their safety and efficacy profile are far from ideal. Treatment with these drugs is always recommended for all patients in acute phase, in case of accidental contamination with sharp-cutting and contact with mucous membranes, congenital Chagas disease, infected mothers of childbearing age, transfusion-related transmission, reactivated infections in immunosuppressed hosts and chronic disease in children younger than 12 years. Both drugs have shown successful results and the parasitological cure of treatment with Bnz, occurs in estimated 80% to 100% of patients during the acute phase, but the effectiveness decreases with advancement of the infection; and the data on adults with late chronic infection indicates serological cure only in 5-20% of cases. The frequency of adverse effects with Nfx is 43.0-97.5% in adults with chronic infection, leading to the discontinuation of the treatment in 14-75% of cases. Compound Bnz is generally preferred over Nfx because of its bet-
of the nitro group to DNA. Previous work of our group showed that the nitro group is not the sole responsible for the genotoxic activity. The type and position of different substituents bonded to the imidazole ring have a significant influence on the toxicological activity. Continuing our work in the search for bioactive substances and knowing that mutagenicity is an undesirable property in clinically used drugs because raises the question of their potential carcinogenicity, more studies are needed for complete evaluation of nitroimidazole effect on DNA, contributing to the elucidation of mechanisms involved in these processes. A nitroimidazole possessing high trypanocidal activity with none mutagenicity is of great interest not only from a safety point of view, but also provides a basis for further investigations of the mode of action and mechanism of expression of mutagenicity of this class of compounds.

In this article we investigate seven nitroimidazole compounds (4-10) analogs to megazol (3) using bioisosterism among rings in order to elucidate the relationship between chemical structure, trypanomicidal and genotoxic activities. In compound 4, the 1,3,4-thiadiazole nucleus presents in 3 was replaced by the 1,2,4-triazole nucleus (Fig. 2); in 5 in addition to replacing the nitro group at the 4-position, plus NH as a spacer group (Fig. 2); in 6, the nitro group was transferred from the 5-position of the imidazole ring of 3 to the 4-position of 6 and the 1,3,4-thiadiazole ring of 3 replaced by the pyrazole ring in the α-position with the nitro group. Ring bioisosterism was also performed to obtain 7, 8, 9 and 10 replacing the pyrazole ring by another azoles with addition of hydrophilic and lipophilic groups respectively, as described in Fig. 2.

The aim of the present study was to evaluate whether the genotoxic effect was abolished in the seven synthesised nitroimidazoles (4-10) analogs to 3, as well as to correlate this effect to their in vitro activity in T. cruzi. The in vitro alkaline comet assay (CA) and the in vitro cytokinesis-block micronucleus assay (CBMN) in whole human blood cells (WHBC) were employed as genotoxicity assays. The in vitro CA is a useful, fast screening system in mammalian cells that can be used in a test battery during drug development. It is widely used in basic research in the pharmaceutical and chemical industry to investigate genotoxic mechanisms as screening assay but it is not preconised for regulatory purposes. The single cell gel electrophoresis (SCGE)
The DNA damage was expressed as percent aberration frequencies. Increased MN frequency in lymphocytes is a recognised predictor of cancer risk.

**MATERIALS AND METHODS**

The compounds 5-10 were prepared as previously reported by our group.\(^{(59,22,32,33)}\)

*In vitro* trypanocidal assay - To accomplishment of the experiments were used bloodstream trypomastigotes form Y strain of *T. cruzi*, obtained at the peak of the parasitemia (7th day after infection) of infected Swiss albino mice.\(^{(34)}\) The trypomastigotes underwent a differential centrifugation process for the separation of erythrocytes, leukocytes and concentration of the parasites in the plasma, and the purified parasites were resuspended in RPMI medium and cell concentration was determined by counting in the Neubauer chamber. Stock solution of each compound was prepared in dimethyl sulfoxide (DMSO), and the assays were performed in Dulbecco’s modified Eagle medium in 96-well plates. In the first well was placed twice the highest desired concentration of each compound, in a final volume of 200 µL, in the following wells were added 100 µL of medium supplemented with 10% foetal bovine serum (FBS) and 2% L-glutamine. Subsequently, 100 µL of parasite suspension (10⁶ trypomastigotes) were added, resulting in a final concentration of 5 x 10⁵ parasites/mL, incubated for 24 h at 37°C under a 5% CO₂ atmosphere and quantified in Neubauer’s chamber by light microscopy. The activity of the derivatives was expressed by the parameter which corresponds to the concentration of the compound that produces 50% lysis of the parasites (IC₅₀). At least four independent experiments were performed and the mean and standard deviation were calculated. Benznidazole (1) was used as a reference drug. The experiments were performed in accordance with the guidelines established by the Oswaldo Cruz Foundation Committee of Ethics for the Use of Animals (L 038/2018).

*In vitro* treatment and cytotoxicity assay in whole human blood cells (WHBC) - Heparinised WHBC was obtained by venipuncture from healthy young non-smoking volunteers with no known recent exposures to genotoxic chemicals or radiation immediately before the assays. WHBC was treated for 2 h at 37°C with different concentrations of compounds 3, 5, 8, 10 (149-10,000 µM) or 4, 6, 7, 9 (149-6,400 µM) in 5% (v/v) DMSO (solvent-control) and then used in the assays. The cytotoxicity assay aims to establish the degree of cell viability after treatment with nitroimidazoles to define the ranges concentrations to be tested in the *in vitro* CA. Cell viability was determined at the end of the treatment using the fluorescein diacetate (FDA)/ethidium bromide (EtBr)-assay, in which viable cells are labelled in green, while dead ones display orange-stained nuclei. WHBC (50 µL) was mixed with an equal volume of the freshly prepared staining solution consisting of 30 µg/mL FDA plus 8 µg/mL EtBr in phosphate-buffered saline (PBS). Samples (50 µL) were spread on a microscope slide and covered with a coverslip and observed using a fluorescence microscope. Two hundred cells were analysed for each treatment.\(^{(32)}\) The research project involving the use of human blood samples was approved by the Committee on Ethics in Research with Human Beings - CEP Fiocruz/IOC (CAAE: 41684815.3.0000.5248) under the consolidated opinion of CEP No. 1066061.

In *in vitro* alkaline comet assay (CA) in whole human blood cells (WHBC) - DNA damage in WHBC was evaluated at the end of 2 h-treatment in duplicate with compounds 3 to 10, at the same concentrations indicated above using the *in vitro* CA. Methyl methane-sulfonate (MMS) (160 µM) (Sigma-Aldrich) was used as a positive control. Aliquots of 5 µL WHBC were mixed with 120 µL of 0.5% low melting-point agarose (LMPA) (Sigma-Aldrich) in PBS at 37°C and were applied to microscope slides (with frosted ends), previously covered with 1.5% normal melting-point agarose (Sigma-Aldrich). Slides were prepared, lysed (pH 10; 4-5°C) and processed as described earlier,\(^{(32)}\) using a time of alkali denaturation of 20 min and electrophoresis (0.86 V/cm and 300 mA) of 20 min at a pH > 13. After the neutralisation, fixation and staining steps\(^{(32)}\) the slides were analysed using a fluorescence microscope at 400 X magnification. Fifty randomly selected cells per slide (200 cells per treatment) stained with EtBr (20 µg/mL) were analysed visually according to tail size into one of four classes of DNA damage: 0 (undamaged, i.e., no visible tail), 1 (slightly damaged), 2 (moderately damaged) and 3 (maximally damaged, i.e, head of comet was very small and most of the DNA in the tail).\(^{(24,32)}\) The DNA damage was expressed as percentage of cells into four classes and as arbitrary units (AU) according to the formula: AU = (0 x n₀) + (1 x n₁) + (2 x n₂) + (3 x n₃), where n = the number of cells analysed in each class. The total DNA damage score in AU (TAU) for 200 cells can range from 0 TAU (200 undamaged cells) to 600 TAU (all cells maximally damaged). Differences between the mean values of TAU from two and three independent experiments under the same conditions, respectively, for each concentration of compounds 4-8,10 and 3, 9 were tested for significance (p < 0.05) in relation to the solvent-control group using Student’s one-tailed t-test. In addition, the effects of compounds 3-10 on the...
intercellular distribution of DNA damage were tested for statistical significance using one-way ANOVA followed by a Dunnett’s multiple comparison test to compare each concentration of the compounds. The computer program GraphPad Prism® sixth version was employed in the statistical analysis of the data.

In vitro cytokinesis-block micronucleus assay (CBMN) with whole human blood cells (WHBC) - Heparinised WHBC samples were obtained by venipuncture from volunteers as described above. The CBMN was performed with WHBC cultures following the OECD guideline 487. WHBC cultures were set up in 10 mL plastic culture tubes (Nunc, Denmark) by adding 0.5 mL freshly collected blood to 4.5 mL of pre-warmed (37°C) 1640 RPMI medium (Gibco, USA) supplemented with 20% FBS (Gibco, USA), 10^5 U/mL penicillin G potassium, 10^2 µg/mL streptomycin sulfate, 3% phytohaemagglutinin M (PHA-M: Gibco, USA) and incubated at 37°C. WHBC cultures were treated for 4 h at 37°C with different concentrations of compounds 3 to 10 (150-10,000 μM) in 5% (v/v) DMSO (solvent-control) 44 h after the start of the cultures. WHBC cultures exposed to mitomycin C (MMC) (1.0 μg/mL in water) (Bristol, USA) for 2 h or to N-deacetyl-N-methylolcholine in water (Demecolcine: Sigma-Aldrich) 0.02 μg/mL for 28 h were used as positive control cultures. After the treatment, the WHBC cultures were centrifuged (900 rpm, 10 min) and washed with 5 mL PBS. After another centrifugation, the cell pellets were resuspended in 5 mL fresh complete RPMI medium as described above, but without PHA-M and with 4.5 μg/mL cytochalasin B (CytB) (Sigma-Aldrich) and incubated at 37°C. CytB has cytokinesis-block activity leading to the formation of binucleate cells. Then, the cultures were harvested at the end of a total culture time of 72 h by centrifugation and treated with 5 mL hypotonic solution (0.56% KCl, 4-6°C) for 10 min and fixed once for 10 min at room temperature with 5 mL methanol/glacial acetic acid (5:1, -20°C) mixed with an equal amount of 0.9% NaCl and then fixed three times with methanol/glacial acetic acid (5:1, -20°C) for 15 min at room temperature. The fixed cell suspension was dropped on a clean glass slide and the slide was air-dried on a heating plate (60°C). Air-dried slides were stained with 60 μg/mL acridine orange in Sörensen buffer (0.03 M KH₂PO₄, 0.03 M Na₂HPO₄) for 3s, then they were embedded in distilled water and covered with a coverslip. MN showing bright green fluorescence were analysed using a fluorescence microscope at 400 X magnification. MN were scored in 2,000 BNC was determined. Cytotoxicity was measured using the cytokinesis-block proliferation index (CBPI) and the replication index (RI) which were calculated from 500 cells. The CBPI indicates the average number of nuclei per cell, and may be used to calculate cell proliferation. The RI indicates the relative number of cell cycles per cell during the period of exposure to cytoB in treated cultures compared to control cultures and can be used to calculate the % cytostasis. CBPI was calculated according to the formula: CBPI = [(No. mononucleate cells) + (2 x No. binucleate cells) + (3 x No. multinucleate cells)] / N, where N indicates the total number of cells scored. RI (%) was calculated according to the formula: RI (%) = (N - No. binucleate cells) / (N + No. multinucleate cells) x 100

where T indicates treated cultures and C control cultures

% cytostasis = 100 - RI

Cytotoxicity was evidenced in the occurrence of reduction in CBPI or RI of cultures treated by test substances when compared to control cultures. The measurement of cytotoxicity was used to select the concentrations of the test substance to be analysed for the presence of MN. The maximum concentration used in MN analysis recommended by OECD 487 in the presence of CytB is the one that induces 45 ± 3% reduction of CBPI or RI when compared to the solvent - control. However, it was adopted in this work as the maximum concentration in CBMN one that induced a proliferation inhibition not exceeding about 50% established by ICH S2 (R1) for lymphocyte cultures.

The chi-square test was performed from a contingency table tabulating the number of BNC with and without MN to test the significance (p < 0.05) of the results of each concentration of compounds 3-10 in relation to the solvent-control culture or of each concentration of positive controls in relation to untreated cultures (control culture). The chi-square test for trend was performed to analyse if the increase of MNC was concentration-related.

RESULTS

In addition to 3, the trypanocidal activity was tested against trypomastigote forms of T. cruzi (Table I). After 24 h of treatment, the compound 10 showed the highest activity with an IC₅₀ = 5.4 ± 0.6 μM, about eight times higher than 9 (IC₅₀ = 45.3 ± 4.0 μM), 48 times higher than 4 (IC₅₀ = 256.8 ± 53.0 μM), 65 times higher than 8 (IC₅₀ = 353.7 ± 27.0 μM). The compounds were selected for testing on trypomastigote forms of T. cruzi (Table I).

| Compounds | IC₅₀/24 h (μM) |
|-----------|-------------|
| 3         | 9.9 ± 0.8⁷  |
| 4         | 256.8 ± 53.0⁹ |
| 5         | > 500      |
| 6         | > 500      |
| 7         | > 2000     |
| 8         | 353.7 ± 27.0⁷ |
| 9         | 45.3 ± 4.0  |
| 10        | 5.4 ± 0.6   |
| Benznidazole | 8.8 ± 1.1  |

a. IC₅₀: concentration that produces 50% lysis of the parasites; b. ref 23; c. ref 32.
353.7 ± 27.0 μM), at least 93 times higher than 5 (IC$_{50}$ > 500 μM) and 6 (IC$_{50}$ > 500 μM) and 370 times higher than 7 (IC$_{50}$ > 2000 μM) (Table I). Compound 10 showed the highest activity, about two times higher than 3 (IC$_{50}$ = 9.9 ± 0.8 μM) and compound 1 (IC$_{50}$ = 8.8 ± 1.1 μM) that is used clinically. Compounds 4-9 were considered inactive molecules based on their IC$_{50}$ values.\(^{(20)}\) Using the FDA/ EtBr assay and in vitro CA, it was investigated the cytotoxicity of compounds 3-10 (Table II) and their capacity to induce DNA damage in WHBC (Fig. 3). Compound 8 (149-10,000 μM) was the only nitroimidazole that did not reduce cell viability. The treatment of WHBC with 3, 5 and 10 (range: 149-10,000 μM) and with 4, 6, 7 and 9 (149-6,400 μM) for 2h at 37°C slightly reduced cell viability (lethality variation range: 1-4%) compared to solvent-control and this effect was not concentration dependent (Table II). All concentrations of the eight compounds studied showed acceptable levels of cytotoxicity, ie, they did not induce values greater than 30% decrease in cell viability when compared to the solvent-control and consequently used in the CA.\(^{(26)}\) The compounds 4\(^{(17)}\) and 7 did not cause DNA strand breaks in the range of 149-6,400 μM compared to the solvent-control group (p > 0.1). However, a significant (p < 0.05) genotoxic effect was observed at concentrations higher than to 610 μM for 5, higher than to 977 μM for 3 and 9, higher than to 1,562 μM for 10, and highly significant (p < 0.01) at concentration higher than 4,000 μM (6,400 μM) and significant (p < 0.05) at 10,000 μM for 8. The genotoxic effect was concentration-dependent for these compounds (Fig. 3) and was not associated with cytotoxicity as shown in Table II. And for compound 6 it was showed a significant (p < 0.05) DNA damage only in the highest concentration tested (6,400 μM). The positive control, 160 μM MMS, induced an extremely significant (p < 0.001) genotoxic effect compared to the control group with a TAU mean value of 554,2 ± 9.9.

In cells treated with 6,400 or 10,000 μM concentrations of compound 3, significant (p < 0.05) class 1 (22.5% and 27.5%, respectively) of DNA damage was observed compared to the percentage of 4.2% showed by the solvent-control group. Compound 9 (6,400 μM) induced significant (p < 0.05) class 1 (40.7%) and highly significant (p < 0.01) class 2 (3.7%) damage compared to 9.0% and 0.7%, respectively for solvent-control. Compound 10 (10,000 μM) caused significant (p < 0.05) class 2 (2.5%) and class 3 (10.2%) damage compared to 0.2% and 2.2%, respectively for solvent-control. Compound 6 (6,400 μM) induced a significant increase of percentage of class 1 (10.5%) DNA damage in relation to 4.8% (solvent-control). In contrast, highly significant (p < 0.01) class 3 (87.7%) damage was observed after treatment with MMS (160 μM) compared to 0% for control culture.

Table III summarises the results of the CBMN with WHBC cultures exposed to different concentrations of compounds 3-10 (150-10,000 μM) for 4 h. With the exception of compound 5, the maximum concentrations used in MN analysis for compounds 3, 4, 6-10, that induced in the maximum 50% reduction of CBPI or RI in relation to solvent-control are indicated in the Table III. All concentrations of compound 5 were considered for MN analysis, because at the highest tested concentration (10,000 μM) there was only a 36% reduction of CBPI and 22% of RI below the maximum acceptable value of 50% reduction.\(^{(25)}\) The maximum concentration of 4,000 μM was established for compounds 3, 6 and 7; 1,600 μM determined for compounds 4 and 10 and 640 μM for compounds 8 and 9. Compounds 5, 6, 8-10 did not induce a significant increase in MN formation when compared to the solvent-control (p > 0.05 for 8 and 10; p > 0.1 for 5, 6 and 9) in the concentration range analysed. It was concluded that these five compounds did not induce chromosomal breaks and/or gain or loss of chromosomes in WHBC. In contrast, a highly significant mutagenic effect (p < 0.01) was observed at concentrations of 1,600 and 4,000 μM for compound 3, significant (p < 0.05) at 1,600 μM for compound 4 and at 4,000 μM for compound 7. When analysed by the chi-square test for trend, the increase in MN formation was concentration-dependent for compounds 3 (p < 0.001), 4 (p < 0.05) and 7 (p < 0.01). The positive control, MMC (1.0 μg/mL) caused an extremely significant clastogenic effect (p < 0.001) inducing in 2,000 BNC, 38 MNC compared to 13 MNC in the control culture and a significant effect (p < 0.05) with 56 and 40 MNC, respectively, compared at 32 and 20 MNC in the control culture. Demecolcine (0.02 μg/mL) showed a highly significant aneugenic effect (p < 0.01) inducing in 2,000 BNC, 37 and 42 MNC, respectively, compared at 13 and 20 MNC (control) and a significant effect (p < 0.05) with 30 MNC compared to 13 MNC in the control culture.

**DISCUSSION**

In the present study it was evidenced for compound 3, a DNA strand break effect through the in vitro CA after 2 h treatment at the concentrations of 1,562, 6,400 and 10,000 μM in the WHBC. In the in vitro CA, the

| Compounds | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------|---|---|---|---|---|---|---|---|
| Control\(a\) | 0.5 | 0 | 0 | 0 | 1.0 | 0 | 0 | 0 |
| Soly-control\(b\) | 6.0 | 0 | 0 | 2.0 | 0 | 0 | 0 | 0 |
| 149 μM | 4.5 | 1.0 | 0 | 0 | 2.0 | 0 | 2.0 | 4.0 |
| 238 μM | 10.0 | 1.0 | 0 | 4.0 | 0 | 0 | 2.0 | 1.0 |
| 382 μM | 1.5 | 1.0 | 0 | 0 | 3.0 | 0 | 4.0 | 2.0 |
| 610 μM | 5.5 | 0 | 0 | 0 | 2.0 | 0 | 0 | 0 |
| 977 μM | 3.5 | 0 | 0 | 2.0 | 2.0 | 0 | 0 | 0 |
| 1,562 μM | 3.0 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2,500 μM | 4.5 | 1.0 | 0 | 6.1 | 0 | 0 | 0 | 0 |
| 4,000 μM | 6.0 | 0.0 | 2.0 | 0 | 0 | 0 | 0 | 0 |
| 6,400 μM | 3.5 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 |
| 10,000 μM | 2.5 | 2.0 | 2.0 | 0 | 0 | 0 | 0 | 2.0 |

*a: ref 32; b: untreated culture; c: 5% dimethyl sulfoxide; results are expressed as percentage decrease (%) in cell viability.*
The highest concentration (10,000 μM) employed corresponds to the maximum concentration to be tested in vitro for relatively non-cytotoxic substances. Similar results were obtained by Boechat et al. and Carvalho et al. who reported a highly significant genotoxic effect for the compound 3 (p < 0.01) on the same test system and experimental conditions for concentrations of 1,562 μM, 2,500 μM and 4,000 μM without reduction of cell viability in the tested range (380-4,000 μM).

The in vitro CA performed on Vero cells, lymphocytes and whole blood was highly sensitive in detecting genotoxicity of 3 at concentrations in the range of 8.8 to 35 μM, well below those employed by Nesslany et al. with the same treatment period of 4 h. Poli et al. showed in fresh leucocytes from rats and mice a dose-response relationship of DNA damage induced by 3. Our results showed that 3 induced chromosomal breaks and/or gain or loss of chromosomes in human lymphocytes evidenced by the highly significant increase of MN at concentrations of 1,600 and 4,000 μM in a concentration dependent manner after treatment period of 4h. The highest concentration of 3 (10,000 μM) was not evaluated for induction of MNs because it caused 63% of CBPI reduction and 62% of RI reduction, higher than the limit of 50% of cytotoxicity recommended for analysis by the ICH S2(R1). Nesslany et al. also reported for 3 provided by Farmanguinhos a high mutagenic activity in the in vitro micronucleus assay in L5178Y mouse lymphoma cells and treatment for 24 h at concentrations of 625 μM and 1,250 μM not associated to cytotoxicity. The compound 3 had its genotoxicity confirmed in other in vitro and in vivo mammalian cell assays. It was a potent inducer of structural chromosome aberrations in vitro in human lymphocytes after 4 h of treatment at the highest concentration possible to be analysed due to cytotoxicity (625 μM); significant increase at the three concentrations (156, 312 and 625 μM) for the treatment of 20 h and at 357 and 625 μM for the 44 h treatment. In the in vivo micronucleus assay in rat bone marrow cells, the two daily doses (two days) given orally (500 and 1,000 mg/kg), with 24 h sampling after the second dose induced a significant increase in frequency of micronucleated polychromatoid erythrocytes in male and female Sprague-Dawley rats. Although 3 is a potent trypanocidal and bioavailable agent when administered orally, its toxicity has led to the discontinuation of its development process for the treatment of Chagas’ disease and sleeping disease.

With the exception of compound 3, which showed in the concentrations 1,562, 6,400 and 10,000 μM, DNA strand break inducer effect and in 1,600 and 4,000 μM clastogenic and/or aneugenic effect in WHBC, all compounds analogs to 3 that were positive in the in vitro CA (5, 6, 8-10) did not induce chromosomal breaks and/or gain or loss of chromosomes. The DNA strand breaks induced by the five analogs (5, 6, 8-10) of 3 in the CA may be repaired, resulting in no persistent effect, and may be lethal to the cell. On the other hand, compounds 4 and 7 that did not induce DNA strand breaks were clastogenic and/or aneugenic in human blood cells, respectively at concentrations of 1,600 and 4,000 μM. Therefore, the clastogenic DNA damage of 4 and 7 was not detected by the in vitro CA.

In relation to compound 6, mutagenicity was reported in the Ames test in TA100 strain of Salmonella typhimurium at the highest tested concentration (50 μg/mL) in the absence and presence of S9 mixture. It showed cytotoxic activity in the concentration of 50 μg/mL in TA98 and in the range of 1.0-50 μg/mL in TA100 and TA1535 strains. Our results of the CBMN of 6 in WHBC, recommended by OECD (2016) differed from

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**Fig. 3: DNA damage induction by nitroimidazoles (3-10) in human whole blood cells using the in vitro alkaline comet assay.** A: megazol (3) (n = 3); B: compound 4 (n = 2); C: compound 5 (n = 2); D: compound 6 (n = 2); E: compound 7 (n = 2); F: compound 8 (n = 2); G: compound 9 (n = 3); H: compound 10 (n = 2). Results are expressed as mean ± standard error mean of total arbitrary units (AU) from two and three independent experiments, respectively for each concentration of compounds 4-8, 10 and 3,9. Only the result of compound 4 has already published (ref. 32). Control corresponds to untreated culture; solvent-control corresponds to 5% dimethyl sulfoxide and positive control to 160 μM methyl methane-sulfonate. For Student’s one-tailed t-test, the asterisks indicate significance at 5% (*) and 1% (**) in relation to the solvent-control and 0.1% (***) in relation to the control.
TABLE III
DNA damage induction by nitroimidazoles (3-10) in human whole blood cells using the *in vitro* cytokinesis-block micronucleus assay

| Compounds | Groups               | CBPI | RI (%) | NMNC/2000 binucleated cells | Chi-square-test (p) |
|-----------|----------------------|------|--------|----------------------------|---------------------|
| 3         | Control               | 1.322| -      | 13                         | -                   |
|           | Solvent-control       | 1.294| -      | 24                         | -                   |
|           | 150 µM                | 1.342| 119    | 24                         | -                   |
|           | 640 µM                | 1.302| 105    | 20                         | -                   |
|           | 1,600 µM              | 1.202| 69     | 47                         | < 0.01 **           |
|           | 4,000 µM              | 1.146| 49     | 50                         | < 0.01 **           |
|           | 10,000 µM             | 1.108| 38     | -                          | -                   |
| 4         | Control               | 1.236| -      | 13                         | -                   |
|           | Solvent-control       | 1.200| -      | 25                         | -                   |
|           | 150 µM                | 1.220| 106    | 28                         | -                   |
|           | 640 µM                | 1.230| 107    | 36                         | -                   |
|           | 1,600 µM              | 1.218| 76     | 44                         | < 0.05 *            |
|           | 4,000 µM              | 1.054| 22     | -                          | -                   |
|           | 10,000 µM             | 1.052| 23     | -                          | -                   |
| 5         | Control               | 1.188| -      | 32                         | -                   |
|           | Solvent-control       | 1.238| -      | 25                         | -                   |
|           | 150 µM                | 1.310| 151    | 20                         | -                   |
|           | 640 µM                | 1.228| 111    | 8                          | -                   |
|           | 1,600 µM              | 1.192| 95     | 20                         | -                   |
|           | 4,000 µM              | 1.340| 167    | 24                         | -                   |
|           | 10,000 µM             | 1.152| 78     | 36                         | -                   |
| 6         | Control               | 1.184| -      | 32                         | -                   |
|           | Solvent-control       | 1.128| -      | 35                         | -                   |
|           | 150 µM                | 1.184| 148    | 32                         | -                   |
|           | 640 µM                | 1.204| 132    | 12                         | -                   |
|           | 1,600 µM              | 1.134| 114    | 16                         | -                   |
|           | 4,000 µM              | 1.068| 56     | 12                         | -                   |
|           | 10,000 µM             | 1.036| 24     | -                          | -                   |
| 7         | Control               | 1.172| -      | 32                         | -                   |
|           | Solvent-control       | 1.082| -      | 17                         | -                   |
|           | 150 µM                | 1.160| 211    | 9                          | -                   |
|           | 640 µM                | 1.170| 220    | 10                         | -                   |
|           | 1,600 µM              | 1.016| 23     | -                          | -                   |
|           | 4,000 µM              | 1.048| 68     | 33                         | < 0.05 *            |
|           | 10,000 µM             | 1.034| 43     | -                          | -                   |
| 8         | Control               | 1.168| -      | 40                         | -                   |
|           | Solvent-control       | 1.200| -      | 36                         | -                   |
|           | 150 µM                | 1.154| 150    | 20                         | -                   |
|           | 640 µM                | 1.108| 69     | 16                         | -                   |
|           | 1,600 µM              | 1.028| 26     | -                          | -                   |
|           | 4,000 µM              | 1.020| 17     | -                          | -                   |
|           | 10,000 µM             | -     | -      | -                          | -                   |
those obtained by Mello et al.\(^{(39)}\) who showed a significant increase of MN in RAW 264.7 cells at concentrations of 10 and 100 µg/mL when treated for a period six times higher than that used in our study.

In the preclinical evaluation of drug candidates, genotoxicity tests are required by regulatory agencies to evaluate the potential risk of cancer induction. Among these tests the CA especially **in vivo**, the CBMN and the **in vitro** micronucleus assay are the most used in the evaluation of the potential risk of cancer induction.\(^{(38)}\) According to ANVISA, in agreement with other internationally recognised regulatory agencies, it is recommended that genotoxicity tests should be completed prior to conducting phase 2 clinical trials.\(^{(38)}\)

The CA allows the investigation of DNA damage in any cell culture or tissue that can be subjected to single cell isolation. Through this technique it is possible to evaluate DNA damage and repair in proliferating and non-proliferating cells at the individual level using extremely small cell samples (5-10 µL). The CA under highly alkaline conditions (pH > 13) during electrophoresis allowed the detection of a broader range of DNA damage.\(^{(28)}\) This includes SSB which may result from direct interaction of the test chemical with DNA or which are related to incomplete excision repair as well as alkali labile sites. As a result, not only clastogenic DNA damage can be detected but also lesions which may give rise to gene mutation.\(^{(38)}\)

The presence of MN in lymphocytes indicates unrepaired damage, from consequences of chromosome mis-segregation or clastogenic events which is manifested after anaphase.\(^{(38)}\) Increased MN frequency in lymphocytes is a recognised predictor of cancer risk in humans and indicates pre-cancerous lesions.\(^{(31)}\)

In the drug evaluation strategy performed, greater relevance should be given to the results obtained in the CBMN than in the **in vitro** CA because the former is considered the standard genotoxicity test in the guidelines for drug evaluation.\(^{(35,41)}\)

It must also be considered that mutagenicity, clastogenicity and aneugenicity are the types of genotoxicity endpoints associated with human disease that should be given the most weight when conducting a human risk assessment. Assays evaluating DNA damage, such as DNA strand breaks in the CA and the measurement of DNA adducts can be useful to determine the presence of DNA damage and can be used to demonstrate an absence of strand breakage and therefore reduced potential to induce heritable alterations. However, their utility for quantitative evaluations is limited because the extent to which DNA damage may be repaired before conversion to a permanent genetic alteration is difficult to ascertain. DNA strand breaks occur during DNA repair and during apoptosis and before necrosis, and so strand breakage may not always be related directly to the formation of mutations or chromosomal aberrations.\(^{(42)}\)

Among the negative substances in the CBMN (5, 6, 8-10), substance 10 was the most promising to proceed with the development as a drug candidate in the treatment of Chagas disease. In addition to the absence of genotoxicity endpoints associated with human disease, substance 10 showed high trypanomicidal activity for *T. cruzi* (IC\textsubscript{50} = 5.4 ± 0.6 µM), about two times higher than 3 (IC\textsubscript{50} = 9.9 ± 0.8 µM)\(^{(23)}\) and 1 (IC\textsubscript{50} = 8.8 ± 1.1 µM) used clinically. Substitution biosisteric of 1,3,4-thiadiazole ring of 3 by lipophilic group linked to azole C-4 and the change from the 5-position nitro group to the 4-posi-

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| Compounds | Groups | CBPI | RI (%) | NMNC/2000 binucleated cells | Chi-square-test (p) |
|-----------|--------|------|--------|-----------------------------|---------------------|
| 9         | Control\(^a\) | 1.250 | -      | 20                           | -                   |
|           | Solvent-control\(^b\) | 1.160 | -      | 36                           | -                   |
|           | 150 µM | 1.188 | 105    | 24                           | -                   |
|           | 640 µM | 1.102 | 60     | 20                           | -                   |
|           | 1,600 µM | 1.034 | 17     | -                            | -                   |
|           | 4,000 µM | 1.022 | 11     | -                            | -                   |
|           | 10,000 µM | 1.060 | 31     | -                            | -                   |
| 10        | Control\(^a\) | 1.208 | -      | 32                           | -                   |
|           | Solvent-control\(^b\) | 1.238 | -      | 25                           | -                   |
|           | 150 µM | 1.174 | 79     | 24                           | -                   |
|           | 640 µM | 1.148 | 58     | 27                           | -                   |
|           | 1,600 µM | 1.122 | 61     | 40                           | -                   |
|           | 4,000 µM | 1.104 | 23     | -                            | -                   |
|           | 10,000 µM | 1.006 | 3      | -                            | -                   |

\(^a\): untreated culture; \(^b\): 5% dimethyl sulfoxide; CBPI: cytokinesis-block proliferation index; RI: replication index; NMNC: number of the micronucleate cells; \(^c\): high cytotoxicity did not allow the calculation of CBPI and RI; the maximum concentrations used in the analysis of micronucleus by inducing in the maximum 50% reduction of CBPI or RI relative to the solvent-control are indicated in bold; for the chi-square test, the asterisks indicate significance of the increase of the NMNC/ 2,000 binucleated cells at 5% (*') and 1% (**') levels in relation to solvent-control.
tion of the imidazole ring in 10 abolished the undesirable mutagenic effect of prototype 3\(^\text{(9)}\) and as a consequence decreasing its effects on the carcinogenicity.\(^\text{(10)}\)

As a follow-up test to evaluate metabolism, pharmacokinetics, and DNA repair of compound 10, the \textit{in vivo} micronucleus assay for the detection of chromosome damage is recommended and performed in immature (polychro-matic) bone marrow erythrocytes of mice or rats.\(^\text{(35,46)}\) And as a second \textit{in vivo} genotoxicity assay to evaluate DNA strand breaks is recommended the \textit{in vivo} CA especially in liver or stomach cells of rodents after oral exposure to compound 10.\(^\text{(35,38)}\) The use of both assays allows also evaluating the systemic or \textit{in situ} genotoxicity.\(^\text{(40)}\)

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**AUTHORS’ CONTRIBUTION**

ACMVT, TRC, RCL and HPSZ - Planned, developed and performed the cytotoxicity assay (fluorescein diacetate/ethidium bromide-assay), \textit{in vitro} alkaline comet assay and \textit{in vitro} cytokinesis-block micronucleus assay with whole human blood cells; BMCSQ, MCSRF, ASC and NB - developed the rational planning and chemical synthesis of nitroimidazole compounds; KS and SLC developed and performed the trypanocidal assays.

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