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Evaluation of *in vitro* toxicity of *N,N*-dimethyl-2-propen-1-amines isomers

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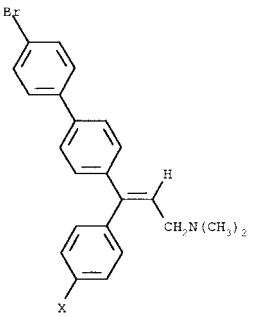
The trypanocidal activities of *cis*-3-(4′-bromo[1,1′-biphenyl]-4-yl)-3-(phenyl)-*N*,*N*-dimethyl-2-propen-1-amine (**Vb**) and *cis*-3-(4′-bromo[1,1′-biphenyl]-4-yl)-3-(4-bromophenyl)-*N*,*N*-dimethyl-2-propen-1-anine (**Vg**) appeared 6.3 and 3.5 fold more active than the *trans*-isomers, respectively. Multi-endpoints for toxicity were also applied. Neutral red uptake (NRU), tetrazolium salt reduction (MTT), DNA content on V79 fibroblast cell culture and acute toxicity von *E. coli* were measured. The IC_{50} through DNA contents was lower for the *cis*-isomers in both series of compounds **5b**: 7.8 μM and **5g**: 5.2 μM). NRU values for derivative **5b** in isomeric mixture shows the same value as the isolated isomers however, in the case of **5g** a more significant toxicity of the *cis*-isomer was found. MTT values show that **5g** is more toxic than **5b**. In both cases, the acute toxicity of the *trans*-isomers was higher than that of the *cis*-isomers.

1. Introduction

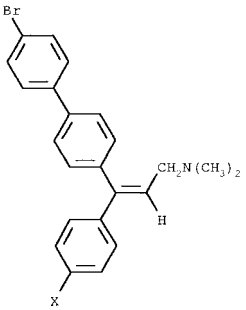
Chagas' disease (American trypanosomiasis) caused by the flagellate *Trypanosoma cruzi*, has an estimated prevalence of around 16–18 million cases in Latin America. One of the most important route of infection is blood transfusion [1]. Only one nitroheterocyclic drug, *N*-benzyl-2-nitroimidazole-1-acetamide (benznidazole), is in clinical use, with a severely restricted applicability for chronic patients. It shows considerable toxicity. The synthesis of several 3-(4′-bromo[1,1′-biphenyl]-4-yl)-3-(4-X-phenyl)-*N*,*N*-dimethyl-2-propen-1-amine derivatives were described [2] as potential trypanocide agents with relative low acute toxicities [3]. The trypanocidal activity of these derivatives on the three evolutive stages of *T. cruzi*, the bloodstream trypomastigote form and both proliferative epimastigote and amastigote forms were studied [3]. For both proliferative forms of *T. cruzi*, total lysis occurred at 10–60 μM and for trypomastigotes at 40–200 μM. The following order or susceptibility was established: amastigotes > epimatigotes > trypomastigotes. The most active compounds were the *p*-bromo (**5g**) and unsubstituted (**5b**) derivatives, which have a 13- and 8-fold higher activity against trypomastigotes than 3-methyl-4-(5′-nitrofurfurilidene-amine)-tetrahydro-4 *H*-1,4-thiazine-1,1-dioxide (nifurtimox) [3].
Cytotoxicity in the Chinese hamster V79 cell line, measured as DNA content of cellular macromolecules, which are indicative of the total cell number, showed that all the compounds present the same range of IC_{50} values (7.0–12.4 μM). The halogen derivative and the unsubstituted one are the less toxic in the series [2, 3]. A multi-endpoint cytotoxicity method using a V79 fibroblastic cell line and acute toxicity studies using *E. coli* were applied to the isomeric mixture of 2-propen-1-amines derivatives and nifurtimox (eliminated by its high toxicity) [4]. In the cytotoxicity analysis of these compounds using this methodology, nifurtimox exhibited a lower toxicity than *N*,*N*-dimethyl-2-propen-1-amine derivatives. Since it is known that nifurtimox is toxic to mammalian cells and that free radicals generation is the base of its toxicity, the fibroblast culture method appears to be an inadequated technique to study the toxicity since prior metabolization is necessary to express this activity [5]. All the *N*,*N*-dimethyl-2-propen-1-amines studied followed the same trends, except for the *p*-NO_2 derivatives which exhibited a higher acute toxicity than those in the others assays, in a similar manner as nifurtimox. Both contain a nitro group and probably act by the same toxicity mechanism. This study led us to select the *p*-bromo-2-propen-1-amine derivative as one of the less toxic and more active trypanocide derivatives for further *in vivo* studies. When a daily dose of 20 $mg \cdot kg^{-1}$ of the *p*-bromo derivative **5g** for 9 consecutive days was used, no parasite was found by optical microscopy [6]. A significant parasitemic decrease was also observed with a single dose (100 $mg \cdot kg^{-1}$) of this compound. Moreover, both treatment schemes displayed a strong protective effect characterized by decreased of mortality [6]. Due to the potentiality of this derivative, further investigation of this class of compounds as chemotherapeutic agents for Chagas' disease is of prime importance.
We now report the isomeric isolation of the unsusbtituted (**5b**) and the *p*-bromo (**5g**) *N*,*N*-dimethyl-2-propen-1-amines, their acute toxicities on an *Escherichia coli* ATCC 25922 strain and their cytotoxicities.

2. Investigations, results and discussion

3-*N*,*N*-dimethyl-2-propen-1-amine derivatives were prepared by Friedel-Crafts reaction between 4-bromo-biphenyl and the corresponding benzoyl chloride to give the ketone followed by a subsequent Wittig reaction with 3-(*N*,*N*-dimethylamino) ethyltriphenyl-phosphonium bromide [2]. The synthesized compounds are shown in the Scheme. Their isomers were isolated and characterized by NMR, MS FTIR and UV spectra.



5g: *Z-trans* = X = Br
5b: *E-trans* = X = H



5g: *E-cis* = X = Br
5b: *Z-cis* = X = H

Acute toxicity on *E. coli* (ATCC 25922 strain) were evaluated through the measurement of CO_2 released by the bacteria during respiration, an indirect parameter of bacteria growth inhibition [7].

These endpoints provide information related to different cellular organelles or cellular compartments. Inhibition of Neutral red uptake (NRU) is an indicator of membrane integrity at the lysosomal level [8, 9]. The reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used to assess the mitochondrial dehydrogenase activity of viable cells [10], and the DNA or protein content is used to evaluate the content of cellular macromolecules, which are indicative of the total cell number [11–13].

The *p*-bromo *N*,*N*-dimethyl-2-propen-1-amine derivative (**5g**) was obtained as shown in the Scheme in an isomeric mixture of an *E-cis/Z-trans* 1 : 1 ratio in 75% yield.

The isolation of isomers were carried out by preparative TLC on Silica Gel 2–25 μm (Aldrich); using chloroform/acetone/hexane (50 : 6 : 44) saturated in ammonium hydroxide aqueous solution as eluent. HPLC separation was achieved in a Waters 600 E Millipore with Hi−Chrom preparative HPLC column, spherisorb S5W 5μ 25 cm × 10 mm.

The best condition for isomer separation was hexane/ethyl acetate/triethylamine (80 : 19 : 1) as eluent in a flow rate of 3 ml/min. Under these conditions, the retention times for the *cis*-isomer and the *trans*-isomer were 63 min and 68 min, respectively. The spectral characterization and the Nuclear Overhauser Effect (NOE) experiments are described in the Experimental section.

Recent results [14] from trypanocidal activity evaluation of these compounds showed that the *Z-cis*-isomer of **5b** and the *E-cis*-isomer of **5g** were 6.3 and 3.5 fold more active against trypomastigote forms than the *trans*-isomers. In order to better understand the toxicity of *N*,*N*-dimethyl-2-propen-1-amines, the multi-endpoints cytotoxicity method was applied. The data set shown in the Table indicate cytotoxicity and acute toxicity on *E. coli*.

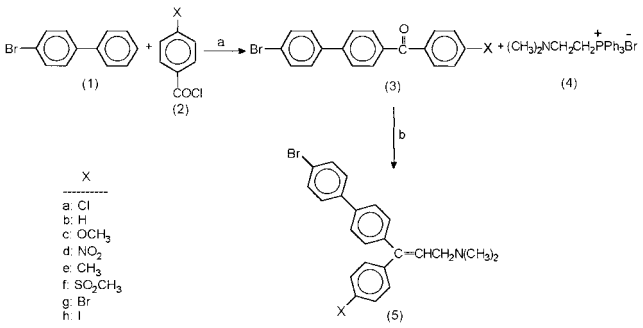
The DNA content is an useful parameter to quantify the cellular material. The cellular growth of V79 fibroblast exposed to different concentrations of *N*,*N*-dimethyl-2-propen-1-amines (X = H, *p*-Br-groups) in its isomer mixture (*E*/*Z*) and isolated isomers were measured.

Fig. 1 shows the dose-response in the DNA content. The IC_{50} values for the *p*-Br derivative **5g** as an isomeric mixture, for *Z-trans* and *E-cis* isomers were 8.4± 0.5 μM, 6.8 ± 0.5 μM and 5.2 ± 0.1 μM, respectively. The isomeric mixture of **5g** showed the best trypanocide activity (13.1 μM) [15] and exhibited a higher relative cytotoxicity determined by the DNA content (IC_{50} = 8.4 μM) than un-

Table: Cytoxicity and acute toxicity of 2-propen-1-amine isomers

2-Propen-1-amines (μM)	NRU (IC_{50})	MTT (IC_{50})	DNA (IC_{50})	Acute toxicity (ED_{50})
X = H *Z*/*E*	5.4 ± 0.9	14.9 ± 0.3	10.8 ± 0.7	6.5 ± 0.8
Z (*cis*)	6.5 ± 0.7	9.1 ± 0.4	5.3 ± 0.6	5.0 ± 0.3
E (*trans*)	6.6 ± 0.6	16.1 ± 0.4	10.7 ± 0.8	3.8 ± 0.1
X = Br *Z*/*E*	11.1 ± 0.2	5.6 ± 0.4	8.4 ± 0.5	2.5 ± 0.5
E (*cis*)	5.9 ± 0.1	6.5 ± 0.1	5.2 ± 0.1	0.7 ± 0.1
Z (*trans*)	7.1 ± 0.7	8.0 ± 0.4	6.8 ± 0.5	2.2 ± 0.2

Scheme



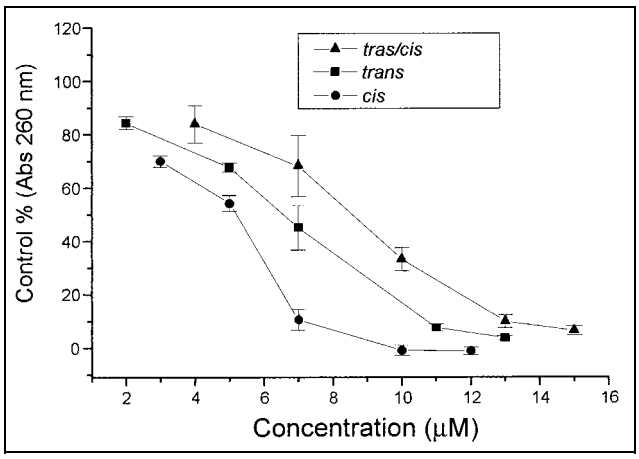


Fig. 1: Dose-response curves in the relative DNA content assay with the isomeric mixture of compound **5g**

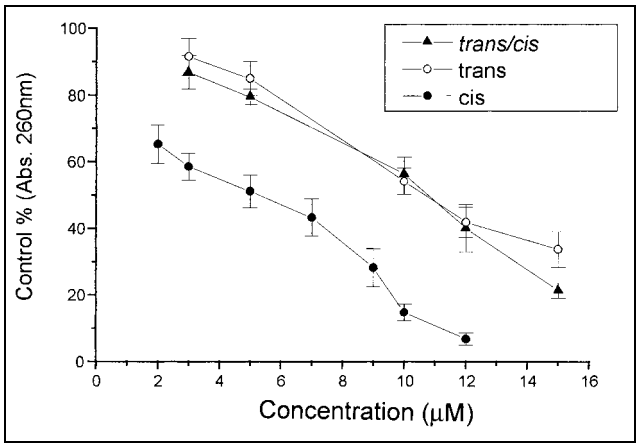


Fig. 2: Dose-response curves in the relative DNA content assay with the isomeric mixture of compound **5b**

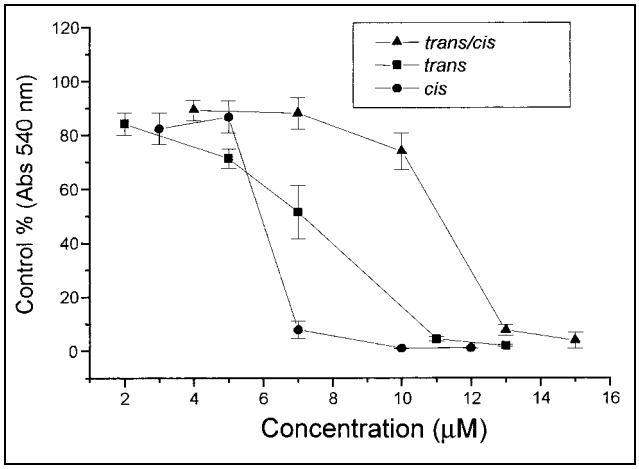


Fig. 3: Dose-response curves in the relative NRU assay with compound **5g** as isomeric mixture, *Z-trans* and *E-cis* form

substituted one ($IC_{50} = 10.8\ \mu M$). The same trend was found for the acute toxicity (EC_{50}).

The *trans*-isomer of the unsubtituted compound **5b** exhibited similar toxicity than the isomeric mixture followed by DNA content analysis (Fig. 2) whereas both isomers of the *p*-bromo substituted derivative **5g** were more toxic than the isomeric mixture. Obviously the interactions in both compounds presumably are different. Metabolization studies of both compounds are in progress to understand this particular behavior.

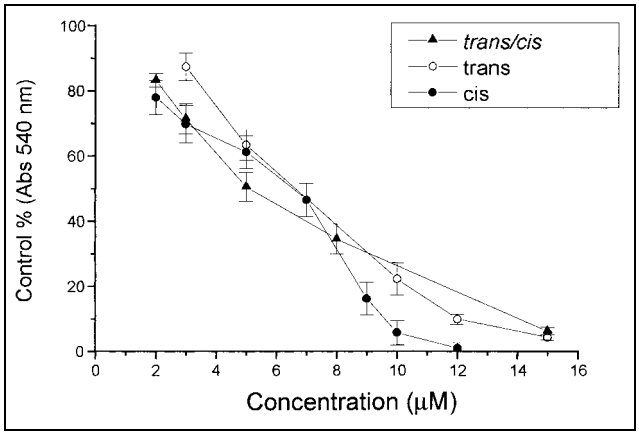


Fig. 4: Dose-response curves in the relative NRU assay with compound **5b** as isomeric mixture, *Z-trans* and *E-cis* form

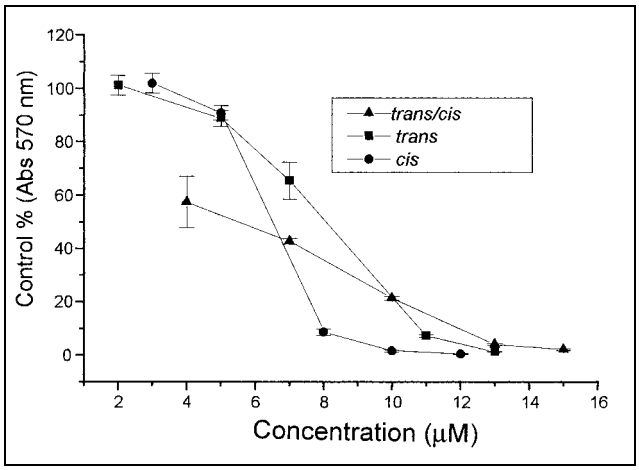


Fig. 5: Dose-response curves in the relative MTT assay with compound **5g** as isomeric mixture, *Z-trans* and *E-cis* form

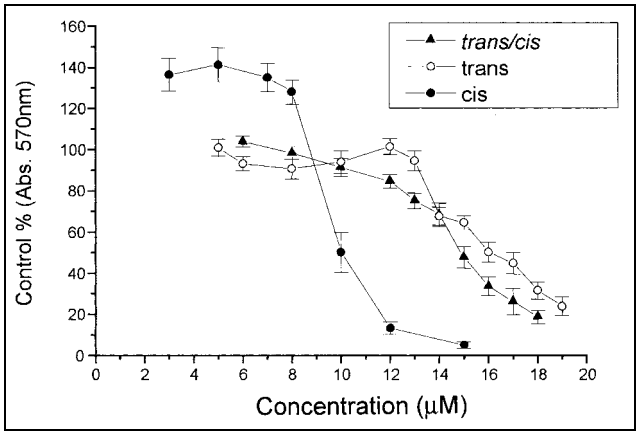


Fig. 6: Dose-response curves in the relative NRU assay with compound **5b** as isomeric mixture, *Z-trans* and *E-cis* form

The cytotoxicity of *N*,*N*-dimethyl-2-propen-1-amines was also measured by the NRU test. Fig. 3 shows a dose-dependent inhibition of stain uptake induced by the *N*,*N*-dimethyl-2-propen-1-amines in the range from 2 to 16 μM for **5g**. The IC_{50} values for the isomeric mixture of the unsubstituted (**5b**) (Fig. 4) and the p-Br-derivative (**5g**) were 5.4 μM and 11.1 μM, respectively. There was no significant difference between the mixture and the isomers of **5b** in the NRU test. However, these compounds were more toxic in the NRU test compared to the DNA and MTT method. This behavior confirms what was described before about the amphiphilic properties of *N*,*N*-dimethyl-2-propen-1-amines, that are probably able to complex between polar lipids and consequently, induce an accumulation of lipids within the lisosomes [4]. The *Z-trans* and the *E-cis* isomer of **5g** exhibited a value of 7.1 μM and 5.9 μM, respectively. The value for *E-trans*- and *Z-cis*- **5b** were 6.6 μM and 6.5 μM, respectively. The *cis*-isomer of **5g** appeared as the most toxic compound at lysosomal level, meaning that this isomer interacts selectively with the lysosomal membrane. The dose-response curve of MTT reduction of **5g** (V79 cells) is presented in Fig. 5. The IC_{50} value for **5g** was 5.6 μM. The *Z-trans* isomer and *E-cis* isomer values were 8.0 μM and 6.5 μM, respectively. The result in this text for **5b** was 14.9 μM for the isomeric mixture. These results indicate that **5g** is more toxic than **5b** (Fig. 6).

Moreover, Fig. 6 shows that derivative **5b** stimulates the MTT reduction at lower drug concentrations. This is probably due to an hyperactivation of the mitochondrial succinic dehydrogenase activity and/or the assessment of cytosolic NAD (P)/NAD (PH) rdox balance [4].

In the acute toxicity test the *trans*-isomer of **5b** was more toxic than the *cis*-isomer. The opposite occurred with **5g**.

In summary, the *E-cis* isomer of **5g** and the *Z-cis* isomer on **5b** were the most active compounds against *T. cruzi*. Toxicity (NRU, DNA content) and acute toxicity were similar. Determinations of lethal doses, *in vivo* trypanocidal activity in mice and QSAR are actually in progress.

3. Experimental

3.1. Isomer isolation and characterization

The prepataive TLC was carried out on Silica Gel 2–25 μm (Aldrich), using $CHCl_3$/acetone/hexane (50 : 6 : 44) saturated in ammonium hydroxide aqueous solution as eluent. Also HPLC separation was achieved by a Waters 600E Millipore model with Hi-Chrom preparative HPLC column, spherisorb S5W 5μ 25 cm × 10 mm.

5g: ^{1}H NMR (300 MHz, $CDCl_3$/TMS, ppm): *E-cis*: 7.60–7.10 (m, 12 H, aromatic); 6.21 (t, 1 H, C=CH, J = 7 Hz); 3.04 (d, 2 H, CH_2N, J = 7 Hz); 2.25 (s, 6 H, $N(CH_3)_2$). *Z-trans*: 7.60–7.14 (m, 12 H, aromatic); 6.28 (t, 1 H, C=CH, J = 7 Hz); 3.00 (d, 2 H, CH_2N, J = 7 Hz); 2.24 (s, 6 H, $N(CH_3)_2$).

^{13}C NMR (75 MHz, $CDCl_3$/TMS, ppm): *E-cis*: 143 (C), 142 (C), 140 (C), 139 (C), 139 (C), 132 (CH) 132 (CH) 131 (CH), 129 (CH), 129 (CH), 129 (C), 128 (CH), 127 (CH), 122 (C), 122 (C), 58 (CH_2), 45 (CH_3). *Z-trans*: 142 (C), 141 (C), 140 (C), 139 (C), 139 (C), 132 (CH), 132 (CH), 132 (CH), 129 (CH), 128 (CH), 128 (CH), 127 (CH), 122 (C), 122 (C), 58 (CH_2), 45 (CH_3).

^{13}C NMR DEPT (75 MHz, $CDCl_3$/TMS, ppm): *E-cis* positive signals: 132 (CH), 132 (CH), 131 (CH), 129 (CH), 129 (CH), 128 (CH), 127 (CH) negative signal: 58 (CH_2). *Z-trans* positive signals: 132 (CH), 132 (CH), 132 (CH), 129 (CH), 128 (CH), 128 (CH), 127 (CH).
negative signal: 58 (CH_2).

IR (film, CH_2Cl_2, cm^{-1}). *E-cis*: 1656 (C=C); 816 e 739 (CH aromatic). *Z-trans*: 1655 (C=C); 806 (CH aromatic)

MS (m/z): *E-cis* 471 ($M^{+\cdot}$), 456 ($M^{+\cdot} - CH_3$), 427 ($M^{+\cdot} - N(CH_3)_2$), 346 (427 −Br), 314 ($M^{+\cdot} - (C_6G_4)$ Br), 265, 238, 189, 165, 82, 70, 58. *Z-trans* 471 ($M^{+\cdot}$), 456 ($M^{+\cdot} - CH_3$), 427 ($M^{+\cdot} - N(CH_3)_2$), 346 (427 −Br), 314 ($M^{+\cdot} - [C_6H_4)$ Be]), 265, 238, 189, 165, 152, 133, 82, 70, 58.

UV (nm, CH_2Cl_2): *E-cis* $\lambda_{max} = 262$ ($c = 3 \times 10^{-2}$ mg/ml). *Z-trans* $\lambda_{max} = 286$ ($c = 3 \times 10^{-2}$ mg/ml).

M.p. *Z-trans*: 131 °C. *E-cis*: oily meterial.

5b: ^{1}H NMR (300 MHz, $CDCl_3$/TMS, ppm): *Z-cis*: 7.1–7.6 (m, 13 H, aromatic); 6.25 (t, 1 H, C=CH, J = 6.7 Hz); 3.1 (d, 2 H, CH_2N, J = 6.7 Hz); 2.3 (s, 6 H, $N(CH_3)_2$). *E-trans*: 7.1–7.6 (m, 12 H, aromatic); 6.3 (t, 1 H, C=CH, J = 6.7 Hz); 3.0 (d, 2 H, CH_2N, J = 6.7 Hz); 2.3 (s, 6 H, $N(CH_3)_2$).
^{13}C NMR (75 MHz, $CDCl_3$/TMS, ppm): *Z-cis*: 144 (C), 142 (C), 140 (C), 139 (C), 138 (CH), 132 (CH), 130 (CH), 130 (CH), 129 (CH), 128 (CH), 128 (CH) 127 (CH), 122 (C), 122 (C), 58 (CH_2), 45 (CH_3). *E-trans*: 144 (C), 141 (C), 140 (C), 140 (C), 139 (CH), 132 (CH), 130 (CH), 129 (CH), 128 (CH), 128 (CH), 127 (CH), 126 (CH), 122 (C), 122 (C), 58 (CH_2), 45 (CH_3).
IR (film, CH_2Cl_2, cm^{-1}): *Z-cis*: 1636.3 (C=C); 816.3 e 736 (CH, aromatic). *E-trans*: 1655.6 (C=C; 812.6 e 739.3 (CH, aromatic) MS (m/z): *Z-cis* 393/391 ($M^{+\cdot}$), 378/376 ($M^{+\cdot} - CH_3$), 349/347 ($M^{+\cdot} - N(CH_3)_2$), 316/314 ($M^{+\cdot} - C_6G_5$), 268, 252, 160, 115, 70. *Z-cis* 378/376 ($M^{+\cdot} - CH_3$), 349/347 ($M^{+\cdot} - N(CH_3)_2$), 314 ($M^{+\cdot} - C_6H_5$), 268, 252, 160, 115, 70.
UV (nm, CH_2Cl_2): *Z-cis* $\lambda_{max} = 264$ ($c = 3 \times 10^{-2}$ mg/ml). *E-trans* $\lambda_{max} = 286$ ($c = 3 \times 10^{-2}$ mg/ml).
M.p. *E-trans*: 123 °C. *Z-cis*: oily

3.2. Cytotoxicity assays

3.2.1. Cell culture

Chinese hamster V-79 lung fibroblasts were grown as previously reported [11, 12].

3.2.2. Cell growth

Cells were seeded at 3×10^4 cells/ml in wells of 24-well plates and incubated at 37 °C in a 5% CO_2 humidified atmosphere. Forthy-eight hours later, semiconfluent cultures were exposed to the test at eight different concentration ranging from 10 to 300 μM for nifurtimox and from 1 to 30 μM for the 2-propen-1-amines derivatives [11]. The cells were then washed twice with PBS solution containing 1 mM calcium ions, fixed with trichloroacetic acid and submitted to alkaline hydrolysis (0.5 M NaOH). The absorbance of the lysate was read at 260 nm in order to quantify nucleic acid. The IC_{50} corresponds to the drug concentration which caused a 50% decrease of DNA content.

3.2.3. Neutral red uptake

V-79 cells (3×10^4 cells/ml) were seeded in 24 well plates and incubated at 37 °C in a 5% CO_2 humidified atmosphere. Forty-eight hourslater, the semiconfluent culture medium was removed and replaced with fresh medium containing nifurtimox (50–450 μM) or 2-propen-1-amine derivatives (5 to 100 μM) and the cells were incubated for further 24 h. The test agent was removed by inverting the multiwell plate and washing the culture with PBS solution. After 3 h of incubation with a serum free medium containing 50 μg/ml neutral red (NR), pre-incubated for 12 h at 37 °C and filtered through a 0.22 μm Millipore membrane before use, the cells were washed with PBS–Ca^{+2} at 37 °C to remove excess unincorporated stain and 1 ml of an aqueous solution of 1% glacial acetic acid and ethanol (50%) was added to each well to fix the cells and to remove the NR from the solution. The plates were then gently shaken for 20 min on a plate shaker and the absorbance of the solution was measured at 540 nm [15].

3.2.4. MTT assay

V-79 cells (3×10^4 cells/ml) were seeded in 24 well plates and incubated until semiconfluence. The culture medium was removed and replaced with a medium containing nifurtimox (100–500 μM) or the 2-propen-1-amines (2.5–20.0 μM). The cells were incubated for an additional 24 h and the culture medium was removed and replaced with a medium containing 0.5 mg/ml of MTT and incubated for 5 h. The culture medium was removed and 1 ml of ethanol was added to each well to solubilize the formazan formed. The plates were gently shaken for 10 min and the absorbance was measured at 570 nm [10].

3.2.5. Acute toxicity assay

e. coli (ATCC 25922 strain) was used in the acute toxicity assay and the method for determination of bacterial cytotoxicity by flow injection analysis [7] proved to be a reliable and rapid assessment of the toxicity of the compounds by analyzing alteration of the amount of CO_2 roduced and trapped in the culture medium.

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