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Trypanocidal nitroimidazole derivatives: Relationships among chemical structure and genotoxic activity

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ABSTRACT

Human American trypanosomiasis is resurgent in Latin Americans, and new drugs are urgently required as current medications suffer from a number of drawbacks. Some nitroheterocycles have been demonstrated to exert a potent activity against trypanosomes. However, host toxicity issues halted their development as trypanocides. As part of the efforts to develop new compounds in order to treat parasitic infections, it is important to define their structure–activity relationship. In this study, 5-nitromegazol and two of its analogues, 4-nitromegazol, and 1-methyl-5-nitro-2-imidazolecarboxaldehyde 5-nitroimidazole-thiosemicarbazone, were tested and compared for in vitro induction of DNA damage in human leukocytes by the comet assay, performed at different pHs to better identify the types of damage. Specific oxidatively generated damage to DNA was also measured by using the comet assay with endonucleases. DNA damage was found in 5-nitromegazol-treated cells: oxidative stress appeared as the main source of DNA damage. 4-Nitromegazol did not produce any significant effect, thus confirming that 4-nitroimidazoles isomers have no important biological activity. The 5-nitroimidazole-thiosemicarbazone induced DNA damage with a higher efficiency than 5-nitromegazol. The central role in the reduction process played by the acidic hydrazine proton present in the thiosemicarbazone group but not in the cyclic (thiadiazole) form can contribute to rationalise our results. Given its versatility, thiosemicarbazone moiety could be involved in different reactions with nitrogenous bases (nucleophilic and/or electrophilic attacks).

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Abbreviations: FPG, formamidopyrimidine glycosylase; ENDOIII, endonuclease III; ALS, alkali-labile site; SB, strand break; TL, total length

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1. Introduction

Chagas' disease, a protozoan infection caused by the kinetoplastid *Trypanosoma cruzi*, is an endemic disease of major concern in Central and South American countries. According to the World Health Organization, an estimated 20 million people are infected with this parasite and about 25% population of Latin America is at risk of being infected [1]. In spite of the large number of infected patients, there are no commercially available drugs with high efficacy that can be used for the treatment of the disease. In the acute, recent or congenital forms of the disease, the most important drugs now available for treatment are: nifurtimox (3-methyl-4-[(5-nitrofurfurylidene)amino]thiomorpholine-1,1-dioxide), a nitrofuran derivative, and benznidazole (2-nitro-*N*-(phenylmethyl)-1H-imidazole-1-acetamide), a nitroimidazole derivative. Specific chemotherapy with these drugs has been indicated for the treatment of the brief acute phase, with a cure rate of 50–70% [2]. Prolonged follow up studies with chronically infected patients, however, show a very low cure rate (8–20%) in subjects treated for more than 10 years [3–5]. In addition, the differences in the susceptibility and natural resistance of a large number of *T. cruzi* isolates to nitroderivatives has also been suggested as an important factor in the low rate of cure in treated chagasic patients [6,7]. Furthermore, both are unsatisfactory because of their serious side effects, which frequently force physicians to suspend treatment [8].

Nifurtimox may not only lead to cellular damage to *T. cruzi*, but also to mammalian tissues through the formation of free radicals as the result of redox cycling [9]. The transfer of electrons from the activated drug then regenerates the parent compound, thereby forming superoxide radical anions that may give rise to other reactive oxygen species such as hydrogen peroxide and the hydroxyl radical.

Benznidazole was found mutagenic in *Salmonella* [10,11]. Several free radical species similar to those produced by nifurtimox were thought to be involved [9]. The drug's mutagenic effect was attributed to the reaction of benznidazole reactive metabolites with the DNA as well as nuclear proteins [12–14]. Furthermore, benznidazole was found to induce chromosomal breaks in *Aspergillus nidulans* [15], and its clastogenic ability was demonstrated on mammalian cells in vitro [16] and in vivo [16–18].

Taking into account the resistance induction in *T. cruzi* and severe side effects of nifurtimox and benznidazole, the development of safer and more effective drugs appears a very urgent matter. On the other hand, safety evaluation for the use of new drugs, before human exposure is permitted, is a socially important task. The standard bioassay in rodents for the assessment of the carcinogenic potentials of chemicals is extremely long and costly and requires the sacrifice of large numbers of animals. For these reasons, many attempts have been made to develop alternative predictive models for evaluating chemical carcinogenicity, ranging from short-term biological assays (e.g., mutagenicity tests) to theoretical models such as structure–activity relationships. Recognition of structural alerts and critical structural factors is a very important scientific advancement since it can contribute to the design of safer chemicals [19]. In this context, the search for new trypanocides should currently be carried out, using as a first step the evaluation through in vitro screening assays.

The nitroimidazole-thiadiazole derivative CL 64855 (2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole, 5-nitromegazol) has a pronounced trypanocidal activity [20,21] that may be due to the triggering of radical production by the compound [20,22,23]. It has been shown [20] that treatment with 5-nitromegazol entails the production of reactive oxygen species: in aerobic conditions, it can induce, as nifurtimox and possibly benznidazole, redox cycling, which could explain its toxic effects, through the production of superoxide radical anion and then hydroxyl radicals [24] that are highly damaging for cellular structures. Other studies [25–27] suggest that 5-nitromegazol trypanocidal activity occurs *via* a different route from those described for nifurtimox and benznidazole.

The genotoxicity of this compound has been assayed in the Salmonella/microsome test [28]. Most nitroimidazoles are mutagenic in bacteria, and this mutagenicity has been attributed to nitroreductases present in these organisms. It has been argued that the lower capacity of mammalian cells to perform nitroreduction decreases the genotoxic risk of 5-nitromegazol. However, some studies [29,30] have demonstrated its genotoxicity in mammalian cells. As part of the efforts to define the structure–activity relationship for developing new compounds in order to treat parasitic infections, attempts were made to assess the cytotoxic and genotoxic activity of 5-nitromegazol and two of its analogues, 4-nitromegazol, and 1-methyl-5-nitro-2-imidazolecarboxaldehyde thiosemicarbazone (5-nitroimidazole-thiosemicarbazone) and to compare the data to those obtained with benznidazole (Fig. 1). In vitro induction of DNA damage in human leukocytes was assessed by the comet assay performed with different protocols to identify different types of damage [31–34]. Since the location of the nitrogroup in position 4 makes the nitroheterocyclic compounds totally inactive against parasite [23], we used 4-nitromegazol to test whether nitrogroup position was also important for the induction of cytotoxicity and/or genotoxicity in human cells. Since some thiosemicarbazone compounds were found to exhibit potent trypanocidal activity [35,36], the genotoxic effect of 5-nitroimidazole-thiosemicarbazone, showing an open structure, was compared to that of 5-nitromegazol, exhibiting a thiadiazole ring after oxidative cyclization reaction [23].

2. Methods and materials

2.1. Chemicals

Reagents for electrophoresis, normal melting point and low melting point agarose, DMSO, ethidium bromide, 5-carboxyfluorescein diacetate, ethyl methane sulfonate, hydrogen peroxide, buffers, and general laboratory chemicals were from Sigma (Sigma–Aldrich Company Ltd., Milan, Italy). Bleomycin was purchased from Rhône-Poulenc Rorer (Milan, Italy). Benznidazole (Rochagan®) was from Roche Brasil (Rio de Janeiro, Brazil). 5-Nitromegazol, 4-nitromegazol, and 5-nitroimidazole-thiosemicarbazone (1-methyl-5-nitro-2-imidazolecarboxaldehyde thiosemicarbazone, CA 4994-21-2) were synthesised by one of the authors (C. Northfleet de Albuquerque) as well as two

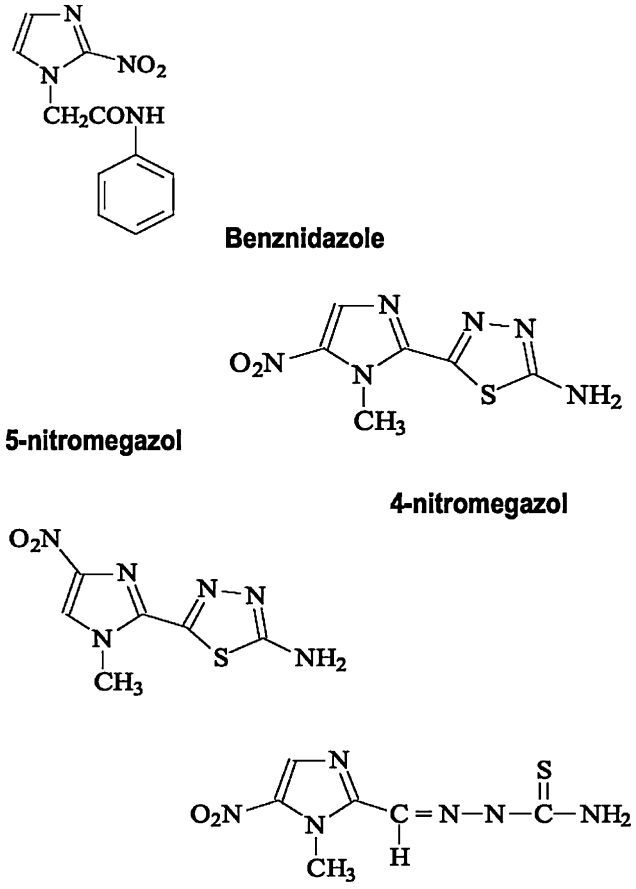

Fig. 1 – Structure of the four nitroimidazoles tested on human leukocytes by the comet assay.

nitrofuran derivatives, 2(1,3,4-thiadiazole)-5-nitrofuran and its thiosemicarbazone, used for further comparisons.

2.2. Isolation and treatment of human leukocytes

Heparin-anticoagulated peripheral blood was obtained by venipuncture from consenting healthy non-smoker donors. In order to isolate the leukocytes, the blood was maintained at 37 °C for 5 min in an erylysis buffer (155 mM NH_4Cl, 5 mM $KHCO_3$, 0.005 mM Na_2EDTA, pH 7.4), centrifuged and washed with PBS, and finally resuspended (~10^6 cells/ml) in RPMI-1640 medium (Gibco).

Appropriate amounts of the compounds were added to an Eppendorf tube containing the cell suspension (10^6 cells) at a final volume of 1 ml. The cells were treated with increasing doses of the compounds for different times (1 h or 5 min) at 37 or 4 °C and then washed twice in PBS. The range of doses was selected in the same range as the plasma concentration of the two nitroheterocycle drugs currently used against Chagas' disease (benznidazole, 2.2–2.8 μg/ml; nifurtimox, 3–6 μg/ml).

2.3. Cytotoxicity assay

Toxicity was checked immediately after exposure. Cell survival was determined by the carboxyfluorescein diacetate/ethidium bromide-assay [37] added with Hoechst 33342 (HO). A freshly staining solution (15 μg/ml carboxyfluorescein diacetate, 2.5 μg/ml ethidium bromide, 2 μg/ml HO in PBS) was prepared. Five hundred microliters of cell suspension (equivalent to about 5×10^5 cells) was mixed with 10 μl of the staining solution, maintained at 37 °C for 5 min. The cells were counted (200 cells per data point) under a fluorescent microscope (DAPI/FITC filters): viable leukocytes, whose nucleus is blue-stained by Hoechst 33342, develop a cytoplasmic green fluorescence, while dead cells accumulate ethidium bromide to develop orange fluorescent DNA.

2.4. Genotoxicity assays

2.4.1. Comet assay

The comet assay was performed only when cell viability had been shown to be ≥70%, according to Singh et al. [31] with minor modifications. Cell lysis was carried out at 4 °C overnight by exposing the cells to a buffer containing 2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10. DNA unwinding was achieved over 40 min in an electrophoretic alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, 0 °C, pH > 13 or pH 12.1), electrophoresis was then carried out for 30 min (0.78 V/cm, 300 mA), at 0 °C in the same buffer, followed by neutralisation in 0.4 M Tris–HCl, pH 7.5. DNA was stained with 100 μl ethidium bromide (2 μg/ml) before the examination at 400× magnification under a Leika DMLB fluorescence microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm) using an automatic image analysis system (Cometa Release 2.1—Sarin). The migration distance between the edge of the comet head and end of the tail (total length, TL) provided representative data on genotoxic effects. The samples were coded and evaluated blind (50 cells per each of two replicate slides per data point). All of the tests were performed at least three times. Ethyl methane sulfonate (2 mM), an alkylating compound, was used as a positive control for the comet assay at pH > 13; bleomycin, a radiomimetic anticancer drug, was selected as a positive control for the comet assay at pH 12.1, since it is a free

Table 1 – DNA damage reported as total length of migration (mean ± S.D. of at least three independent experiments) detected by the comet assay performed at different pH (>13 or 12.1) in human leukocytes treated with different compounds used as positive controls for different cell treatment conditions (37 °C, 1 h or 0 °C, 5 min)

	Ethylmethane sulfonate (2 mM)	Bleomycin (100 μg/ml)	Hydrogen peroxide (50 μM)
Cell treatment	37 °C, 1 h	37 °C, 1 h	0 °C, 5 min
pH > 13	67.51 ± 4.16		40.51 ± 1.61
pH 12.1		62.17 ± 5.40	36.36 ± 0.72

radical-based DNA damaging agent that also induces DNA strand breaks [38] (Table 1).

Additionally, after processing the slides in the comet assay, the occurrence of cells showing completely fragmented chromatin (generally named as 'hedgehogs' or 'clouds' or ''ghost cells'', i.e. non-detectable cell nuclei which represent cellular toxic events) was assessed as a further indicator of cytotoxicity. These cells were not evaluated by image analysis but were recorded separately.

2.4.2. *Modified comet assay for detection of oxidised bases*

By using DNA glycosylases/endonucleases, endonuclease III (ENDOIII) and formamidopyrimidine glycosylase (FPG), able to recognise and cleave classes of lesions, specific DNA base modifications, such as modified purine and oxidised pyrimidine bases, respectively, are converted to strand breaks. These strand breaks can be detected by the comet assay (pH 12.1 or pH > 13). Oxidatively generated damage can be evaluated easily by comparing the DNA migration in enzyme treated and buffer treated slides.

The comet assay, with the modification of an extra step after lysis in which DNA is digested with the repair enzymes, was performed according to Collins et al. [33,39]. Briefly, after cell lysis, the slides were washed three times with the enzyme buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.2 mg/ml bovine serum albumin, pH 8 with KOH) and incubated with ENDOIII, which converts oxidised pyrimidine bases to single strand breaks, or FPG, which recognizes altered purine bases, in this buffer (or in buffer alone as control, detecting only strand breaks and alkali-labile sites). Hydrogen peroxide (50 μM), an inducer of oxidative stress, was used as a positive control (Table 1). ENDOIII and FPG were isolated from bacteria containing over-producing plasmids (Collin's Laboratory, Rowett Research Institute, Bucksburn, Aberdeen, UK).

Since previous papers [34,40] reported that the presence of more alkaline resistant lesions was better detected at pH > 13 than pH < 12.5, we used two different pH (pH 12.1 and pH > 13) for unwinding and electrophoresis solutions. The enzyme-treated gels reveal alkali-labile sites and/or strand breaks (ALS/SB) and oxidised bases (ALS/SB + OX). Assuming a linear dose response, subtraction of (ALS/SB) from (ALS/SB + OX) gives a measure of oxidised bases.

The SPSS 11 (SPSS Inc., Chicago, IL, USA) statistical package was used to analyse statistical differences between samples. Statistical differences between controls and treated samples were first determined with the non-parametric Wilcoxon rank-sum test for each experiment. The mean values from the repeated experiments were used in a one-way analysis of variance. If significant *F*-values ($p < 0.05$) were obtained, Dunnett's *C* multiple comparison analysis and Student's *t*-test were conducted.

3. Results

3.1. *Cytotoxicity assay*

For all compounds, the cellular survival rate did not appear to be affected by the treatment in the range of doses used and independently by time and temperature of exposure when measured immediately after the exposure (data not reported).

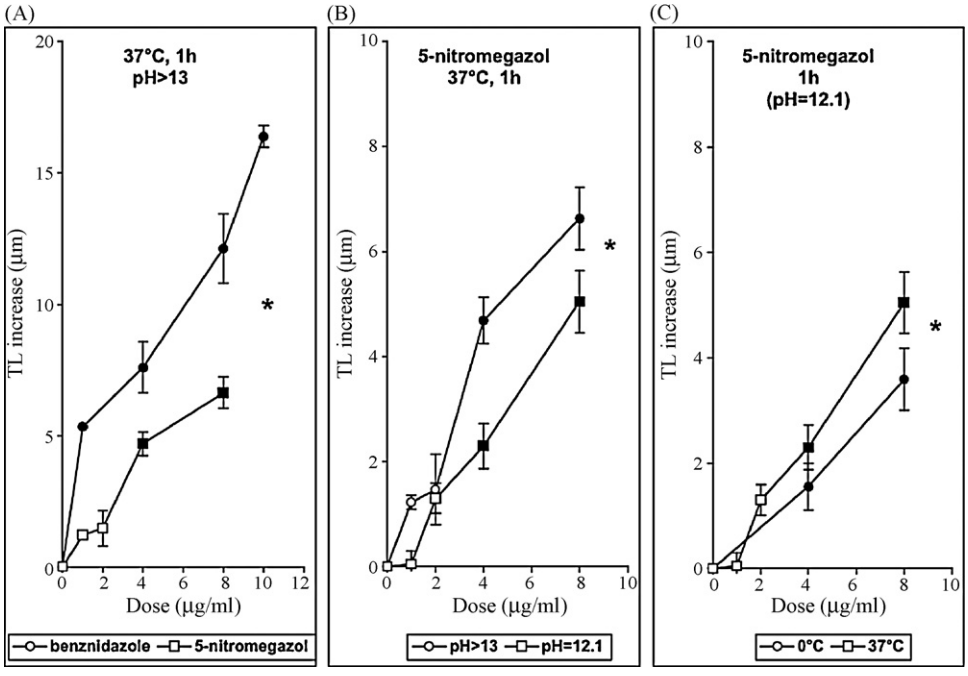

Fig. 2 – DNA damage detected by the comet assay (pH > 13 or pH 12.1) in human leukocytes treated with benznidazole (37 °C, 1 h) and 5-nitromegazol (37 or 0 °C, 1 h). DNA damage is expressed as DNA migration increase (μm) with respect to dose 0 (mean ± S.D. of three independent experiments). Filled symbols, $p < 0.05$ with respect to dose 0. $^{*}p < 0.05$ between the treatments.

Table 2 – DNA damage reported as total length of migration (mean ± S.D. of three independent experiments) detected by the comet assay performed at different pH (>13 or 12.1) in human leukocytes treated with 5-nitromegazol at 0 °C for 5 min

	0 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
pH > 13	32.33 ± 1.10	31.78 ± 1.73	31.60 ± 1.05	31.39 ± 0.34	32.44 ± 0.97
pH 12.1	34.24 ± 1.19	35.37 ± 1.11	34.15 ± 0.88	34.03 ± 0.86	33.96 ± 1.09

The lack of toxicity has been confirmed by the absence of a significant induction of cells showing completely fragmented chromatin in the comet assay (data not reported).

3.2. *Genotoxicity assays*

Human leukocytes treated for 1 h at 37 °C with 5-nitromegazol and, for comparison, with benznidazole, the nitroimidazolic compound usually employed against Chagas' disease, were analysed by the comet assay (pH > 13) for the induction of genotoxicity (Fig. 2A). Both the compounds significantly increased DNA migration, with benznidazole being more active ($p < 0.05$) than 5-nitromegazol.

DNA migration was detected on 5-nitromegazol-treated leukocytes with the comet assay performed at both the pHs (>13 or 12.1) to distinguish different kinds of DNA damage [34,40]. In cells treated with 5-nitromegazol for 1 h at 37 °C, there was detected a significantly greater increase ($p < 0.05$) of DNA migration at pH > 13 than at pH 12.1 (Fig. 2B). These findings suggest that 5-nitromegazol is able to produce both DNA strand breaks and alkali-labile sites, the latter being better detectable at the highest pH [34].

The treatment with 5-nitromegazol was also performed on cells maintained on ice (5 min or 1 h) to minimise cellular processing of possible oxidative damage [39,41]. Oxidatively generated damage to DNA takes the form of single-strand breaks and, to a lesser extent, alkali-labile sites and several kinds of oxidised purine and pyrimidine bases [39,42]. Single-strand break rejoining is generally rapid, with a half-time for repair of a few minutes in normal cells [43,44]. Our results (Fig. 2C) showed how DNA damage induction was related to treatment temperature. The comparison of the data obtained at pH 12.1 on cells treated for the same time (1 h) at different temperatures (37 or 0 °C) shows a significant greater DNA migration (Student's $t < 0.05$) in high temperature-treated cells than low temperature-treated ones. These findings may suggest that DNA strand breaks directly induced by 5-nitromegazol were detectable for the low temperature treatment, whereas the excess of strand breaks observed for the treatment performed at physiological temperature could derive from repair mechanisms. Nucleotide excision repair (NER) that leads to the formation of gaps and that is in general slower than base excision repair (BER) could be more probable. This suggests that bulky adducts could be the targets of the

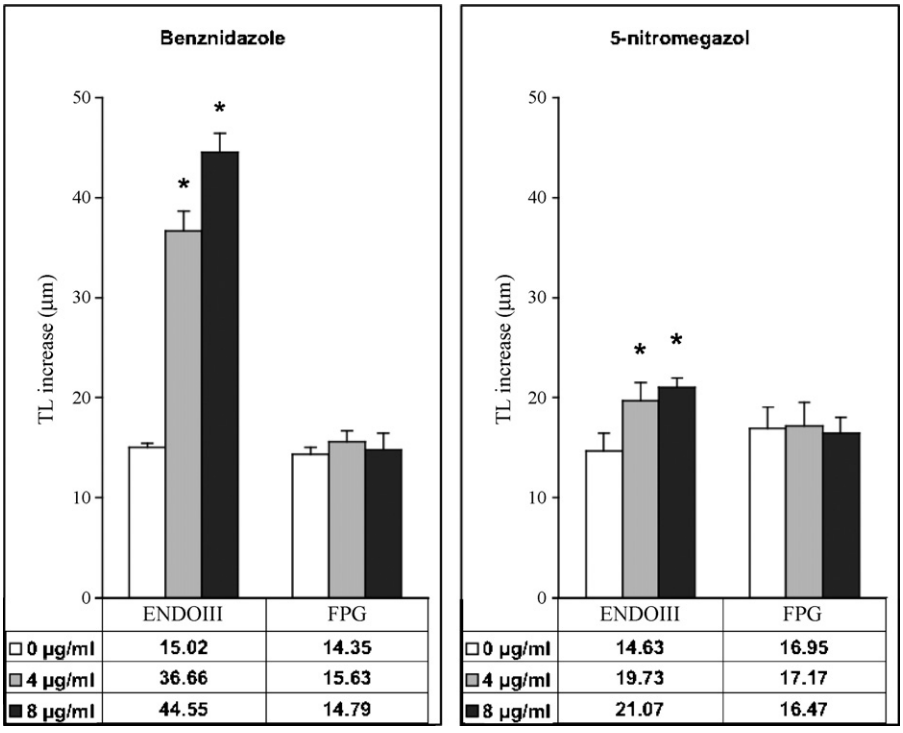

Fig. 3 – DNA damage detected by the comet assay (pH > 13) in human leukocytes treated (37 °C, 1 h) with benznidazole or 5-nitromegazol. Specific oxidatively generated damage to DNA is detected by the comet assay (pH > 13) modified for detection of oxidised bases with the use of bacterial repair endonucleases, ENDOIII and FPG. Oxidised bases are expressed as DNA migration increase (μm) with respect to the assay without enzymes (mean ± S.D. of three independent experiments). * $p < 0.05$ respect to dose 0.

NER process. Using mammalian cells defective in specific repair activities could easily check the involvement of DNA repair enzymes. On the other hand, the formation of DNA strand breaks may not only be due to excision repair enzymes but also to delayed radical production subsequent to damage to mitochondria.

The induction of DNA damage appeared to be also dependent on the treatment time. While a significant increase of DNA migration was observed in cells treated at low temperature (0 °C) after a 1 h exposure (Fig. 2C), no genotoxic effects were detected in short-time (5 min) exposed cells (Table 2), in either experimental conditions used for the comet assay (pH > 13 or pH 12.1).

To improve our understanding of the drug action mechanism, we proceeded to specifically measure oxidatively damaged DNA by using bacterial repair endonucleases in the modified comet assay protocol [33].

A large amount of oxidised pyrimidine bases was found in leukocytes treated for 1 h at 37 °C with benznidazole or 5-nitromegazol (Fig. 3), with a significantly ($t < 0.05$) higher effect

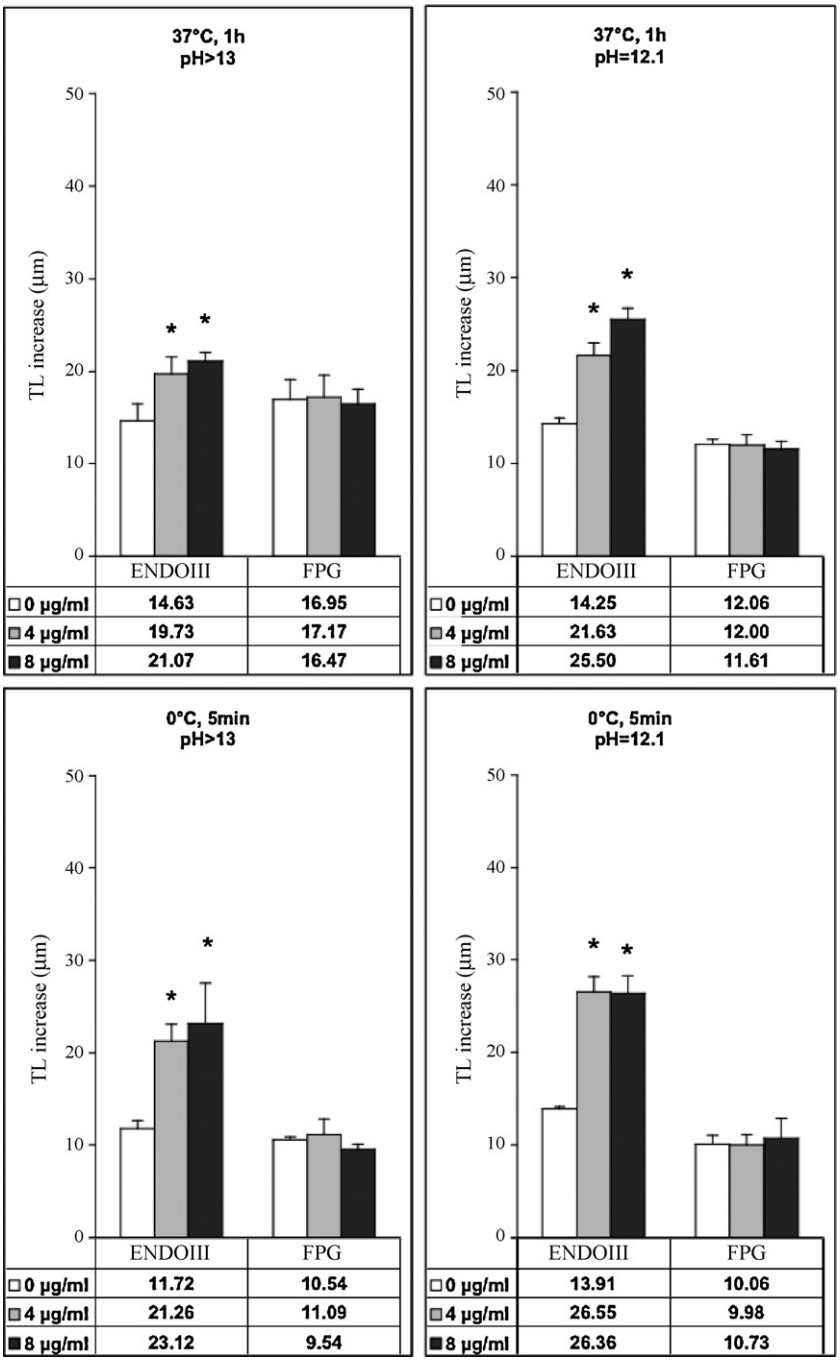

Fig. 4 – DNA damage detected by the comet assay (pH > 13 or pH 12.1) in 5-nitromegazol-treated cells (37 °C, 1 h or 0 °C, 5 min). Specific oxidatively generated damage to DNA is detected by using bacterial repair endonucleases, ENDOIII and FPG. Oxidised bases are expressed as DNA migration increase (μm) with respect to the assay without enzymes (mean ± S.D. of three independent experiments). * $p < 0.05$ respect to dose 0.

in cells exposed to benznidazole (comet assay with ENDOIII, pH > 13).

Specific oxidatively generated damage induced by 5-nitromegazol was also measured at pH 12.1 and analysed in relation both to the time and temperature of treatment (Fig. 4). FPG procedure detected basal oxidative stress but did not yield a significant increase of oxidised purine bases after 5-nitromegazol treatment. Hydroxyl radicals should produce similar amounts of FPG- and ENDOIII-sensitive sites in cellular DNA, whereas our experimental procedures were able to detect only oxidised pyrimidine bases. This fact could be ascribed to the differences between the activities of the two bacterial endonucleases used, which showed a different yield of oxidised bases in cells treated with 50 μm H_2O_2 (DNA migration increase with respect to dose 0 was 42.93 ± 5.66 μm for ENDOIII and 14.69 ± 2.45 μm for FPG). For the 1 h at 37 °C treatment, the presence of oxidised pyrimidine bases was better detected ($t < 0.05$) by using ENDOIII at pH 12.1 than at pH > 13 [34]. As seen above, we did not measure any significant increase of DNA migration in cells treated for 5 min at 0 °C by using the "classic" comet assay. The sensitivity was strongly increased by using the comet assay with the modification in which DNA is incubated with ENDOIII (Fig. 4). The data obtained after the short (5 min, 0 °C) exposure are similar to those detected with the comet assay at pH 12.1 for long exposure both performed at 37 °C (Fig. 4) and 0 °C (Fig. 5). These findings could suggest that 5-nitromegazol acts by at least two time dependent mechanisms: an early oxidation of DNA bases, i.e. similar data for 5 min and 1 h exposures when using ENDOIII, and a later DNA strand breakage, i.e. DNA migration increase detected by the "classic" assay only in cells treated for 1 h, irrespective of the treatment temperature. However, if hydroxyl radical was the main reactive species generated by 5-nitromegazol metabolism, it is not clear why mostly oxidised bases were produced after a short-time exposure, the formation of strand breaks and/or alkali-labile sites requiring apparently a longer period. In fact, hydroxyl radical is able to both oxidise bases and induce strand breaks within DNA, the latter process being more efficient than the former one. So, the observed time dependence effects cannot be solely ascribed to the reaction of 5-nitromegazol generated hydroxyl radicals with DNA. Furthermore, the development of DNA strand breaks may be due to a possible delayed production of hydroxyl radicals after the induction of damage to mitochondria. Further studies should be performed to better define the action mechanisms of 5-nitromegazol.

The 5-nitroimidazole-thiosemicarbazone, immediately preceding 5-nitromegazol in the synthetic pathway, and 4-nitromegazol, 4-isomer of 5-nitromegazol, were tested on human leukocytes for 1 h at 37 °C. The cells were then analysed by the comet assay, both at pH 12.1 and pH > 13 (Table 3). 4-Nitromegazol did not appear to induce a significant increase of either DNA strand breaks or alkali-labile sites, whereas the 5-nitroimidazole-thiosemicarbazone was found significantly ($p < 0.05$) more genotoxic than 5-nitromegazol, with effectiveness at doses ≥1 μg/ml.

The ability of thiosemicarbazone moiety to induce DNA damage was further investigated by testing with the comet assay 2(1,3,4-thiadiazole)-5-nitrofuran and, for comparison, its

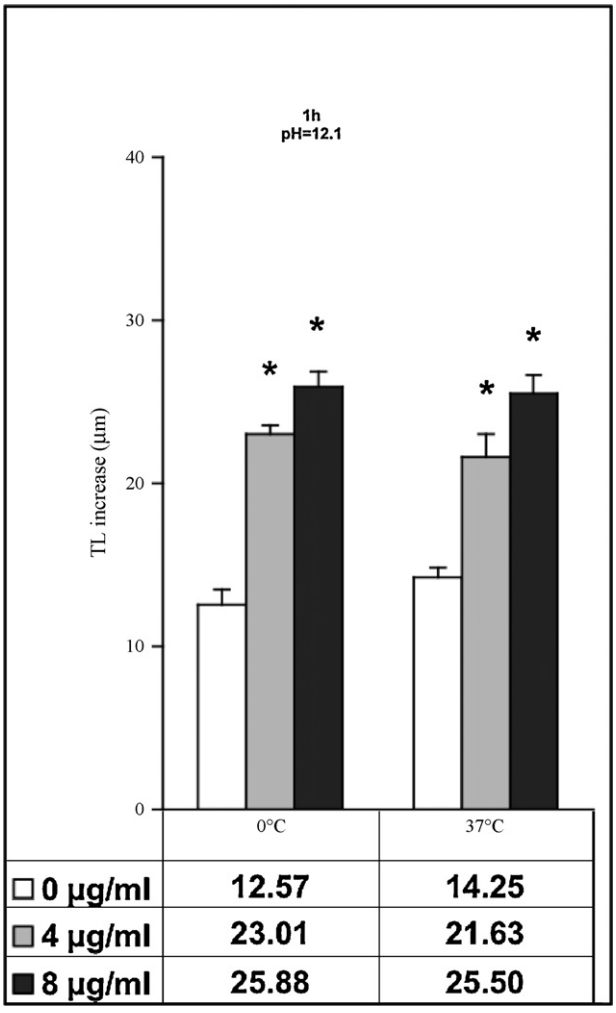

Fig. 5 – Specific oxidatively generated DNA damage detected by the modified (ENDOIII) comet assay (pH 12.1) in leukocytes treated with 5-nitromegazol for 1 h at 0 or 37 °C. Oxidised bases are expressed as DNA migration (μm) increase with respect to the assay without enzymes (mean ± S.D. of three independent experiments). *$p < 0.05$ respect to dose 0.

thiosemicarbazone (Fig. 6). While the 5-nitrofuran derivatives were significantly ($p < 0.05$) less genotoxic than the corresponding 5-nitroimidazoles, it was confirmed that the thiosemicarbazone moiety was more genotoxic than the corresponding thiadiazole ring. These findings, which show how lateral substituents widely modify 5-nitrocompounds' genotoxic potential, are in agreement with previous studies [45] on 5-nitrofuran derivatives and DNA damage induction.

The comet assay protocol modified for specifically detecting oxidised DNA bases (pH 12.1) showed that 4-nitromegazol did not induce any significant increase in the level of damaged bases, whereas the 5-nitroimidazole-thiosemicarbazone was able to produce oxidatively generated damage both in pyrimidine and purine bases (Fig. 7). The 5-nitroimidazole-thiosemicarbazone effects were more pronounced for pyrimidine bases than for purine bases. The results obtained with 5-nitroimidazole-thiosemicarbazone showed a significantly

Table 3 – DNA damage reported as total length of migration (mean ± S.D. of three independent experiments) detected by the comet assay performed at different pH (>13 or 12.1) in human leukocytes treated (37 °C, 1 h) with 5-nitroimidazole-thiosemicarbazone or 4-nitromegazol

Dose (μg/ml)	5-Nitroimidazole-thiosemicarbazone		4-Nitromegazol	
	pH > 13	pH 12.1	pH > 13	pH 12.1
0	31.80 ± 1.30	32.70 ± 1.71	31.44 ± 1.48	33.19 ± 2.48
1	36.49 ± 1.48*	37.16 ± 0.42*	31.60 ± 0.75	32.05 ± 1.13
2	36.18 ± 1.01*	37.80 ± 2.03*	30.60 ± 1.51	34.35 ± 1.09
4	38.57 ± 3.13*	39.12 ± 2.24*	32.37 ± 1.19	32.91 ± 2.22
8	55.24 ± 0.53*	47.53 ± 2.14*	31.96 ± 1.41	33.94 ± 1.90

* $p < 0.05$.

(Student's t, $p < 0.05$) higher damage on DNA bases than in cells treated with 5-nitromegazol.

4. Discussion

The new possible trypanocidal drug 5-nitromegazol was shown to react with DNA [29,30], inducing both DNA strand breaks and alkali-labile sites in in vitro treated human cells. The comparison of data obtained by the comet assay performed with or without the bacterial repair endonuclease ENDOIII (Table 4) clearly show that 5-nitromegazol genotoxic mechanism acts through the induction of oxidised DNA bases, also formed after a very short-time exposure, as well as through the induction of DNA strand breaks/alkali-labile sites. This is in agreement with the hypothesis of radical production by the compound in aerobic conditions [20,22,23]. The relatively high yield of ENDOIII-sensitive sites appears to be a good indicator of the significant implication of hydroxyl radicals [42]. DNA strand breaks could be generated by hydroxyl radical-mediated hydrogen abstraction within the sugar moieties [42]. Another 5-nitroimidazole compound, namely metronidazole, when tested in human lymphocytes using the comet assay, was found to produce, in aerobic conditions, significant dose related increases in DNA damage, whereas application of an antioxidant protected against drug-induced DNA damage [46]. Concerning the mechanisms of action, it is proposed [46] that 5-nitroimidazol compounds induce DNA damage through the so-called futile cycle, i.e. one electron reduction of the drug leads to the production of nitro radical anions which in the presence of oxygen are oxidised and may generate reactive oxygen species (ROS).

ROS production is still further supported by the high ability for the 5-nitroimidazole derivative thiosemicarbazone to induce oxidatively generated damage to both pyrimidine and purine bases. The treatment with the 5-nitroimidazole-thiosemicarbazone induced DNA damage in human leukocytes with a higher efficiency than 5-nitromegazol as shown by our results obtained by the comet assay both with or without ENDOIII (Table 4). When the thiosemicarbazone

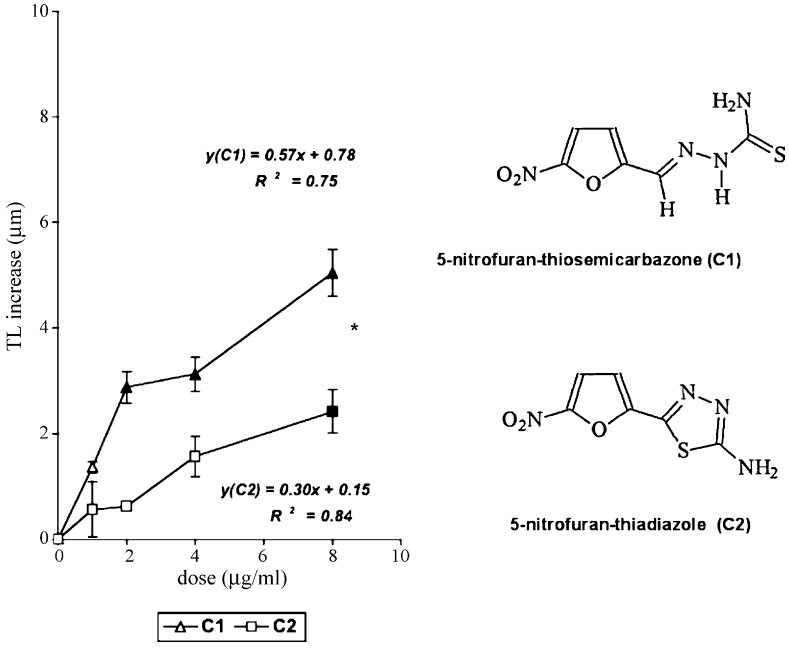

Fig. 6 – DNA damage detected by the comet assay (pH > 13) in human leukocytes treated (37 °C, 1 h) with 5-nitrofuran-thiosemicarbazone (C1) and 5-nitrofuran-thiadiazole (C2). DNA damage is expressed as DNA migration increase (μm) with respect to dose 0 (mean ± S.D. of three independent experiments). Filled symbols, $p < 0.05$ with respect to dose 0. * $p < 0.05$ between the treatments.

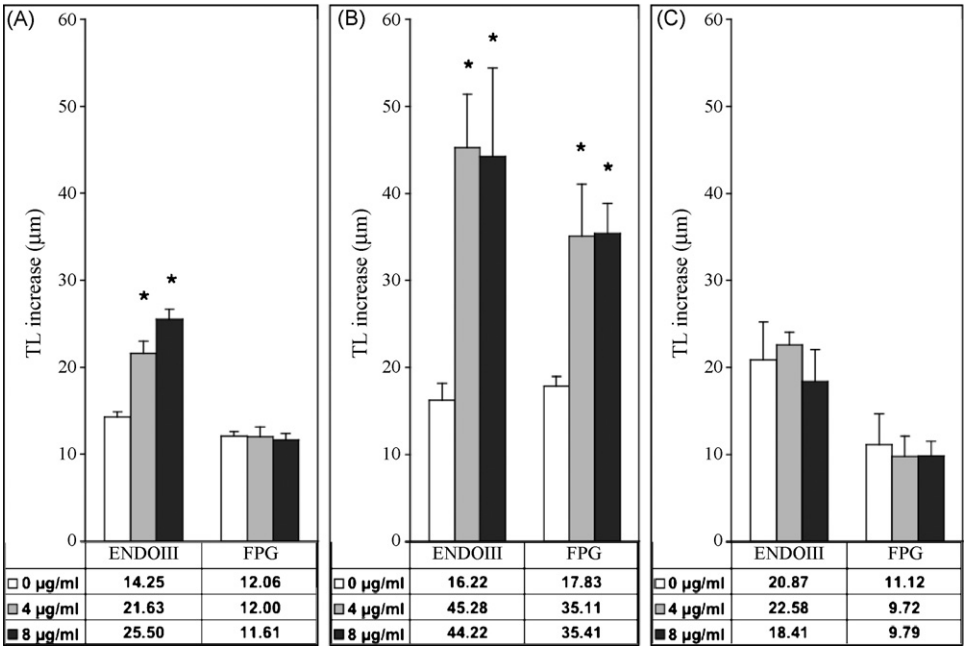

Fig. 7 – DNA damage detected in cells treated (37 °C, 1 h) with 5-nitromegazol (A), 5-nitroimidazole-thiosemicarbazone (B), and 4-nitromegazol (C). Specific oxidatively generated damage to DNA is detected by the comet assay (pH 12.1) modified for detection of oxidised bases with the use of bacterial repair endonucleases, ENDOIII and FPG. Oxidised bases are expressed as DNA migration (μm) increase with respect to the assay without enzymes (mean ± S.D. of three independent experiments). $^{*}p < 0.05$ respect to dose 0.

structural feature is absent, i.e. after oxidative cyclization, a less active compound was afforded, as shown by the results obtained by the comet assay in leukocytes treated with 5-nitrofuran thiadiazole and, for comparison, with its thiosemicarbazone. This suggests that the thiosemicarbazone moiety is an important structural feature contributing to the genotoxic potential. In fact, in a recent electrochemical and ESR study of these compounds [47], it has been reported that nitrofuran thiosemicarbazones show a higher capacity to be reduced and, hence, a better ability to generate radical species with respect to the parent compound (nifurtimox). The thiosemicarbazone moiety confers a remarkable stability to the radical anion that is formed under reduction through an extended delocalisation and, moreover, a central role in the reduction process is reported to be played by the acidic hydrazine proton of the thiosemicarbazone group that is present in the linear but not in the cyclic form. All these considerations, and in particular the latter, contribute to rationalise the facts that we observe in our experiments. Nevertheless, given the versatility of the thiosemicarbazone moiety, also other types of reactions with nitrogenous bases can be envisaged such as nucleophilic and/or electrophilic attacks that could account for other kinds of DNA damage.

In agreement with previous studies [48], which reported genotoxic activities as dependent on the nitrogroup position, our findings show that the 4-nitroimidazole, ineffective against *Tripanosoma* [23], is also unable to induce any detectable DNA damage in the range of doses used. This suggests that some 5-nitromegazol mechanisms of action against the parasite are the same that act on the DNA of mammalian cells.

Our results are in line with the reported clastogenic potential of the 2-nitroimidazole benznidazole previously found both in vivo (increase of fragile sites and micronuclei in

Table 4 – Specific genotoxic activity (DNA migration increase/dose unit calculated by linear regression analysis) of 5-nitromegazol and its thiosemicarbazone on in vitro treated human leukocytes

Compound	Treatment	SB	OX
5-Nitromegazol	5 min, 0 °C	−0.09 ($r^2 = 0.00$)	1.56 ($r^2 = 0.71$)
	1 h, 0 °C	0.45 ($r^2 = 0.59$)	1.66 ($r^2 = 0.89$)
	1 h, 37 °C	0.65 ($r^2 = 0.82$)	1.41 ($r^2 = 0.93$)
5-Nitromegazol thiosemicarbazone	1 h, 37 °C	1.66 ($r^2 = 0.86$)	3.50 ($r^2 = 0.61$)

Comet assay (pH 12.1) performed without (DNA strand breaks, SB) or with bacterial repair endonuclease ENDOIII (DNA oxidised bases, OX). OX are calculated by considering the DNA migration increase with respect to the assay without enzymes. Times (1 h or 5 min) and temperatures (0 or 37 °C) of cell treatments are reported.

patients [17] or chromatid breaks and acrocentric chromosomes in mice [18]) and in vitro (SCE and MN) [16]. The induction of oxidised DNA bases by benznidazole agrees with the production of active oxygen species [24,26]. It is interesting to point out that benznidazole, normally used in Chagas' disease therapy, is more able than 5-nitromegazol to induce DNA strand breaks/alkali-labile sites (about two-fold more active) as well as oxidised DNA bases (more than four-fold).

In conclusion, on account of the intrinsic tendency of nitrocompounds to be genotoxic, further careful studies on the role of chemical moieties such as the nitrogroup and lateral substituents and more targeted vectorisation towards trypanosomes are necessary for the development of safer and more effective drugs.

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