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Cytotoxic and genotoxic effects of megazol, an anti-Chagas' disease drug, assessed by different short-term tests

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Abstract

Cyto- and genotoxicity induced by drugs can limit the dose and duration of treatment, can adversely affect patient quality of life, and may be life-threatening. Two drugs are currently being used for treatment of the acute phase of Chagas' disease and both have serious undesirable effects. In this research, cyto- and genotoxic activity of the nitroimidazole-tiadiazole derivative CL 64855 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole (megazol), a promising alternative drug, was evaluated *in vitro* with different short-term tests: (a) induction of recombination events and mutation in the yeast *Saccharomyces cerevisiae* D7 strain, with and without induction of cytochrome P-450; DNA damage (single and double strand breaks, alkali-labile sites, etc.) by the Comet assay in different mammalian cells. *S. cerevisiae* did not show a significant increase of mutant and recombinant event frequency, both with and without cytochrome P-450. On the other hand, the cytochrome complex appeared to detoxify the drug with respect to cytotoxicity. Results in rat and mouse fresh leukocytes showed a dose–response relation of drug-induced DNA damage. Findings in treated VERO cells suggested a complex treatment time–DNA damage relationship and the possible induction of repair mechanisms. Furthermore, bleomycin effects were increased in rat cells by simultaneous administration of megazol. Megazol shows different biological activity in relation to cellular types and experimental conditions (with or without cytochrome P-450, short/long time of exposure, with or without other genotoxins), thus suggesting a modulation of effectiveness by different physiological/biochemical conditions of cells. The findings could be useful to evaluate new megazol-derived compounds and to assess the risks/benefits relationship for each drug.

Keywords: Comet assay; DNA damage; Mutagenicity; Yeast; Anti-trypanosomal drug; Synergetic effects; Nitroimidazole

1. Introduction

Human trypanosomiasis is a public health problem in many tropical countries and American trypanosomiasis (Chagas' disease) is an endemic parasitic disease afflicting more than 20 million persons in Latin America. As for now, the chemotherapy of Chagas' disease is unsatisfactory, with few substances reaching clinical trial. A better understanding of the action of the currently used drugs and of newly synthesised compounds is necessary in order to identify the mechanisms of their toxicity for the parasite and minimise damage in host cells. With this approach more effective and safer drugs can be obtained.

Chemotherapy can be associated with severe side effects. Cyto- and genotoxicity induced by drug can limit the dose and duration of treatment, can adversely affect patient quality of life, and may be life-threatening. Side

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Abbreviations: NFX, nifurtimox; BZ, benznidazole; MZ, megazol; SCGE, single cell gel electrophoresis; LMA, low melting point agarose; PM, point mutation; GC, gene conversion; MCO, mitotic crossing over; TA, total aberrations; DMSO, dimethyl sulphoxide; PBS, phosphate buffer saline; LDR, length/diameter ratio; FDA, fluoresceine diacetate; EtBr, ethidium bromide; P450, cytochrome P-450; HYC, hycanthone; 2AF, 2-aminofluorene.

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effects, together tissue toxicity, also include long-term effects such as alterations to the immune system and evidence of malignancies, probably due to the genetic damage induced by drug treatment and/or its co-toxic, co-mutagenic, co-carcinogenic and promoting potential.

Various nitroimidazoles are widely used in humans for the treatment of infections with anaerobic organisms. However, some of these drugs show severe side effects. The degree of genotoxicity of nitroimidazoles appears to be affected by various parameters such as aerobic or hypoxic/anaerobic cell condition [1–5], presence of the microsomal fraction S9 mix [1–3,5], presence of exogenous/cellular anti-oxidant and conjugant compounds [4,6–8]. Nevertheless, their data do not completely resolve the parameters, and the drug action appears to be strictly related to drug characteristics [6,9–16].

Chemotherapy of Chagas' disease is still very unsatisfactory. Two drugs are currently being used: 4-[(5-nitrofurfurylidene)amino-3-methylthiomorpholine-1,1-di oxide], nifurtimox (NFX), and *N*-benzil-2-nitro-1-imidazole acetamide, benznidazole (BZ), which act through the induction of oxidative or reductive damage of the parasite. However, NFX and BZ have serious undesirable toxic effects, which have been reported during their clinical use [17]. Furthermore, mutagenicity was shown [2,3,17,18]. These compounds have been used for treatment of the acute phase, but their efficacy varies according to the geographical area, due to differences in drug susceptibility among different strains of *Trypanosoma cruzi*, the causative agent of Chagas' disease.

Taking into account the resistance development in *T. cruzi* and severe side effects of NFX and BZ, the nitroimidazole-tiadiazole derivative CL 64855 2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole, megazol (MZ), might represent a promising alternative.

Detection of the megazol nitro anion radical is possible in the presence of NADPH and ferredoxin: $NADP^+$ oxidoreductase under anaerobic conditions [19]; the radical anion formed in the first step can be transformed into the corresponding nitroso derivative, a compound with known specificity against DNA. Furthermore, the nitroso form can easily react with a secondary and tertiary amino compound generating *N*-nitroso compounds, the most broadly acting and the most potent group of carcinogens [20]. MZ was also shown to undergo bioreduction, yielding the corresponding nitro anion radical [21]. By contrast, Tsuhako *et al.* [19] did not detected the megazol nitro anion radical in the presence of either rat liver microsomes or cellular fractions of *T. cruzi* under conditions where the corresponding NFX anion radical was observed.

The genotoxicity of this highly active compound against several strains of *T. cruzi* has been assayed only in the *Salmonella*/microsome test [22,23]. MZ proved to be a potent mutagen to the frame-shift indicator tester strains TA98 and TA102. No activity was observed with the base-pair substitution mutagen indicator strain TA100 in spot tests. No significant increase in the number of induced mutants could be detected in the presence of rat-liver microsome fraction. The excision-repair-deficient strain TA98 was much more sensitive to the killing action of MZ than TA102, a repair-proficient strain. Most nitroimidazoles are mutagenic in bacteria and this mutagenicity has been attributed to nitroreductases present in these organisms, although the nature of the ultimate mutagens has not been established. Undoubtedly, the ability of microorganisms to reduce these compounds to toxic intermediates is a key part of the clinical effectiveness of nitroheterocyclic drugs. It has been argued that the lower capacity of mammalian cells to perform nitroreduction places animals at a lesser risk to the genotoxic effects of these agents. However, several nitroheterocyclics are mutagenic in mammalian cells and carcinogenic in rodents, so that the protection afforded by the lower nitroreductase capacity of mammalian cells may not be complete [24].

In light of the present knowledge, further studies on the genotoxic potential of megazol appear to be an important goal. On the basis of a better understanding of the mode of action of MZ, more active and safer drugs could be developed. Lack of information is particularly serious for this anti-Chagas' disease drug. Furthermore, registration of a pharmaceutical compound requires a comprehensive (*in vitro* as well as *in vivo*) assessment of a possible genotoxic potential [25,26]. In case of *in vitro* positives, besides the *in vivo* bone marrow micronucleus test, guidelines request a second *in vivo* test. As recently reported [27], the Comet assay is becoming established as a sensitive and easy-to-perform test and accepted by regulatory authorities/ethical committees both for *in vitro* and *in vivo* testing of genotoxic pharmaceuticals.

In this research, megazol was assessed for its genotoxicity in different short-term tests, under aerobic conditions:

- Induction of recombination events (gene conversion and mitotic crossing over), aberration, and point mutation in the yeast *Saccharomyces cerevisiae* D7 strain [28], with and without induction of P450;
- DNA damage (single and double strand breaks, alkalolabile sites) by the single cell gel electrophoresis (SCGE or Comet assay) in fresh rat leukocytes treated *in vitro*.

Living organisms are exposed every day to a multitude of physical, chemical, and biological agents. Assessment of possible deleterious outcomes from these exposure is often concentrated on single agents and neglects the potential for combined effects, i.e. synergism or antagonism. In this context, the possible co-genotoxic potential of megazol by SCGE was evaluated in fresh rat leukocytes treated *in vitro*.

To generalise the data obtained on rat cells to other mammalian cells, we also performed MZ treatment *in vitro* on fresh mouse leukocytes and cultured VERO cells, widely used for *T. cruzi* research [29,30], to assess DNA damage by SCGE.

2. Materials and methods

2.1. Chemicals

Reagents for electrophoresis, normal melting point and low melting point agarose, and general laboratory chemicals were obtained from Sigma. Bleomycin (Bleomicina) was purchased from Rhône-Poulenc Rorer. Megazol was synthesised by one of the authors (Dr. Northfleet de Albuquerque).

2.2. Yeast assays

Strain D7 characteristics:

Genotype :	a	ade2-40	trp5-12	ilv1-92
	α	ade2-119	trp5-27	ilv1-92

Phenotype: trp^-, ile^-.

Spontaneous frequencies:

Gene conversion (*trp5*)	0.4–0.8 × 10^{-5} surviving cells
Mutation (*ile1-92*)	0.3–0.8 × 10^{-6} surviving cells

The diploid strain D7 of *S. cerevisiae* [28] was used to determine the reversion frequencies at the *ilv1-92* mutant (PM) and induction of mitotic gene conversion (GC) at the *trp5* locus with or without endogenous activation. Mitotic crossing over (MCO) was visually detected as pink and red twin sectored colonies, which are due to the formation of homozygous cells of the genotype *ade 2-40/ade 2-40* (deep red) and *ade 2-119/ade 2-119* (pink). Red, red-white, pink and pink-white colonies has been also detected, deriving from point mutation, mitotic gene conversion, deletion, and aneuploidy. All the events linked with ADE 2 locus are defined as total aberrations (TA).

As an alternative system to the microsomal assay, yeast cells were harvested during the logarithmic phase of growth in 20% glucose at maximum activation of cytochrome P-450 complex [31,32].

Both with and without endogenous metabolic activation, the cells (10^8 cells/mL) were inoculated in phosphate buffer 0.1 M, pH 7.0, in the presence of different (at least five) concentrations of testing samples, and kept in an alternating shaker (110 rpm) at 37° for 2 hr. The high temperature was chosen above the normal physiological conditions of the yeast to completely inhibit cell division during the treatment to avoid survival titre mistakes. As suggested by Zimmermann *et al*. [28] and Parry and Parry [33], for the treatments it is essential that the cells are adequately buffered at pH 7.0, as yeast cultures rapidly acidify their media.

After treatment, the harvested cells were plated on a solid complete medium (2% glucose), to determine survival titre and total aberrations, and on selective mineral media to detect gene conversion and mutant reversion frequencies, respectively.

A least squares linear regression analysis was used to calculate specific genotoxic activity (mutants or recombinant per microgram of drug, referred to the treated population of 10^8 cells). DMSO (62.5 μL/mL) was used as a negative control; 2-aminofluorene (5 μg/mL) and hycanthone (100 μg/mL) were used as positive controls when P450 was respectively induced or not.

For all the assays the data were analysed using the modified 2-fold rule [34] in which a response is considered positive if the average response for at least two consecutive dose levels was more than twice the spontaneous frequencies and were subjected to multifactor analysis of variance by computer assistance (SPSS 10 statistical package).

2.3. Single cell gel electrophoresis

2.3.1. Fresh mouse/rat leukocytes

Fresh whole blood of Balb/C mice or Wistar rats was centrifuged, maintained for 10 min in an erylyse solution for leukocyte isolation (155 mM NH_4Cl, 5 mM $KHCO_3$, 0.05 mM Na_2EDTA, pH 7.4) at 37°, washed with PBS and resuspended (~10^6 cell/mL) in PBS. Medium containing cells and appropriate volumes of megazol (five doses: 0, 1, 2, 4, 8 μg/mL) were added to an Eppendorf tube. Bleomycin treatment (50 and 100 μg/mL) was performed as positive control.

Rat cells were also treated in parallel with bleomycin (50 μg/mL) and five doses of megazol (0, 1, 2, 4, 8 μg/mL) to evaluate nitroimidazole co-genotoxicity potency.

Treatments were carried out for 1 hr at 37° and an aliquot was used for SCGE. The assay was performed basically according to Singh *et al*. [35] (cell lysis: overnight, unwinding time: 20 min; electrophoresis time: 20 min, pH 13).

Immediately before the examination, the DNA was stained with 100 μL ethidium bromide (10 μg/mL). The samples were examined under a fluorescent microscope, equipped with an excitation filter BP 515–560 nm and a barrier filter LP 580 nm. All slides, including those of the positive and negative controls, have been independently coded before microscopic analysis and scored without knowledge of the code.

2.3.2. Cultured VERO cells

VERO cells, a selected strain derived from kidney fibroblasts of African "green monkey" (Cell Bank, Instituto Adolfo Lutz, São Paulo, Brazil), were cultured in a humidified incubator at 37° with 5% CO_2 in RPMI-1640 supplemented with 10% (v/v) foetal bovine serum previous inactivated at 56° for 30 min (FCS, Cultilab, Campinas, Brazil), penicillin 200 U/mL, streptomycin 2.5 mg/mL, gentamycin 44 mg/mL, $NaHCO_3$ 2 g/L, *N*-(2-hydroxyetil)-piperazine-*N*′-2-etansulfidric acid 2.38 g/L.

DMSO-dissolved MZ (or DMSO) was added to cultures. Treatment was continued during different prefixed times (1, 2, 4, 20, 40 hr).

After treatment, trypsine 0.2% and EDTA 0.02% solution was added to cultures to separate the cells. These were than centrifuged (10,000 rpm, 2 min), washed in PBS, centrifuged, resuspended in LMA and placed on the first agarose layer of slide (10^4–10^5 cells per slide). Subsequent steps were performed as in leukocytes SCGE.

2.3.3. DNA damage evaluation

In the absence of an automatic system, the evaluation of genotoxic effect was first obtained as visual perception of DNA damage. A number of 100 randomly selected cells per sample were visually scored according to tail intensity. It means that the frequency of round nuclei, nuclei with short tails, nuclei with medium long tails, and nuclei with long tails has counted, categorising nuclei as undamaged (i.e. no migration), short migration, medium migration, long migration. Thus the damage score for each sample can range from 100 (completely undamaged cells: 100 cells × 1) to 400 (maximally damaged: 100 cells × 4). Furthermore, damaged cells percentage was calculated, independently by damage class. Cells with complete DNA migration were defined as "ghost cells." When present, these cells are not used in statistical treatment of the data.

A measure (arbitrary units) of the migration length and diameter of the Comet head (measured perpendicularly to the direction of the electric field) was also performed on every saved Comet image and the length/diameter ratio (LDR) was calculated. Results are presented as frequency distributions of single cell DNA damage in each DNA damage class.

A SPSS 10 statistical package was used (GLM model). Differences in the extent of DNA damage between the control and the treatments were tested for significance using ANOVA analysis of variance with Dunnett's C multiple comparison test (Bonferroni correction) and the data were compared using the non-parametric Kolmogorov–Smirnov test.

2.3.4. Cell viability test

The viability of rat and mouse cells was determined at the end of each treatment using the fluoresceine diacetate (FDA)/ethidium bromide (EtBr) assay [36]. A freshly prepared solution consisting of 30 μL FDA in acetone (5 mg/mL), 200 μL EtBr in PBS (200 μg/mL), and 4.8 mL PBS was used. 40 μL of cell suspension (equivalent to about $4-8 \times 10^5$ cells, i.e. the pellet of remaining cellular suspension after the Comet assay procedure) was mixed with 10 μL of the staining solution, maintained at 37° for 5 min in an Eppendorf tube, twice washed in PBS (1 mL), and centrifuged (7800 *g*, 1 min). The cells were spread on a microscope slide, covered with a coverslip, and observed under a fluorescent microscope (FDA/EtBr blue filter). Viable cells appear green-fluorescent, whereas orange-stained nuclei indicated dead cells: 200–1000 cells were counted per data point.

VERO cells were seeded in 24 well plates for 24 hr, and incubated with 25 μM megazol (diluted in DMSO, final DMSO concentration 0.25%). Untreated VERO cells and cells exposed to 0.25% DMSO were included as controls. Cells were then treated for 1, 2, 3, 24, and 48 hr then immediately washed and incubated with a 0.2% solution of Trypan blue (Sigma) in RPMI medium with 10% FCS for 15 min at 37°. The dye was rinsed and after adding culture medium to the wells, viable and not-viable cells (with blue nuclei) were immediately scored under phase contrast light microscopy.

3. Results

3.1. Yeast

Megazol toxicity on yeast was detected in cells both with and without P450 induction, i.e. logarithmic (log) and stationary (stat) phase, respectively. The data (Fig. 1) indicate cytotoxicity relationship with megazol doses in "stat" cells whereas a detoxifying action of P450 was shown in "log" cells.

Genotoxicity tests were performed in different experimental conditions in which both "stat" and "log" cells were treated with increasing drug concentrations to assess convertant and mutant induction (Table 1). The D7 strain did not show any significant change in revertant and convertant frequencies regardless of cytochrome P-450 presence/absence. The induction of aberrants, as defined in Section 2, also did not appear to be affected by megazol treatment, both with and without cytochrome P-450 induction (Table 2): the dose–response relationship is not significant.

3.2. Rat leukocytes

Cell viability (Table 3), measured immediately after MZ treatment, was always ≥70% in the used dose

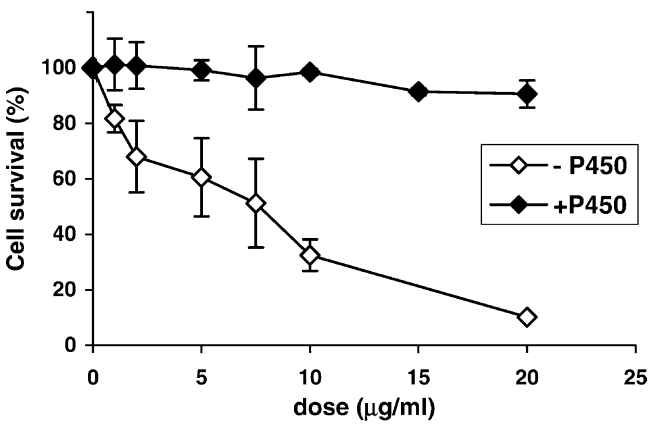


Fig. 1. Megazol toxicity in *S. cerevisiae* D7 strain during stationary growth phase cells (−P450) or logarithmic growth phase cells (+P450). The average survival and standard deviation for each tested dose are indicated.

Table 1
Megazol-induced convertants and mutants in the D7 strain in stationary and logarithmic growth phases

Dose (μg/mL)	Stationary growth phase		Logarithmic growth phase	
	GC/10^5 surviving cells	PM/10^6 surviving cells	GC/10^5 surviving cells	PM/10^6 surviving cells
0	0.80 ± 0.02	0.79 ± 0.06	1.12 ± 0.01	0.66 ± 0.03
1	0.76 ± 0.06	0.83 ± 0.03	1.13 ± 0.01	0.63 ± 0.02
2	0.76 ± 0.02	0.85 ± 0.08	1.18 ± 0.04	0.63 ± 0.01
5	0.79 ± 0.03	0.73 ± 0.08	1.21 ± 0.03	0.58 ± 0.04
7.5	0.79 ± 0.05	0.77 ± 0.08	1.17 ± 0.03	0.65 ± 0.08
10	0.77 ± 0.04	0.77 ± 0.04	1.25 ± 0.15	0.67 ± 0.04
HYC (100 μg/mL)	6.43 ± 0.54	3.53 ± 0.42	–	–
2AF (5 μg/mL)	0.87 ± 0.07	0.71 ± 0.04	27.81 ± 4.25	38.12 ± 5.78

GC: gene conversion at the *trp5* locus; PM: reversion frequencies at the *ilv1-92* mutant. Positive controls; HYC: hycanthone, 2AF: 2-aminofluorene. The means and standard deviations of three independent experiments are shown.

Table 2
Induction of MCO and TA colonies in the D7 strain by increasing doses of megazol, without (stationary) and with (logarithmic) induction of P450

Growth phase	Dose (μg/mL)	Total colonies	Colony phenotype[a]						MCO[b] ($\times10^{-4}$)	TA[c] ($\times10^{-4}$)
			RP	RPW	R	RW	P	PW		
Stationary	0	7542			1		2		0	3.98
	1	6165							0	0
	2	5685			1	2	2	1	0	10.55
	5	5178			2		2	2	0	11.59
	7.5	4530	1	2	1	2			6.62	13.25
	10	2586							0	0
Logarithmic	0	6594			1		1	1	0	4.55
	1	6654	3	3	1	1		1	9.02	13.53
	2	6633	1	2		2	1		4.52	9.05
	5	6546							0	0
	7.5	6330			2	3	3	1	0	14.22
	10	6498	2	1	2	1			4.62	9.23

[a] The abbreviations RP, RPW, R, RW, P, and PW refer to red-pink, red-pink-white, red, red-white, pink, and pink-white colonies, respectively.
[b] MCO: mitotic crossing over represented by RP (twin-spot) and RPW colonies.
[c] TA: total aberrant colonies includes RP, RPW, R, RW, P, and PW colonies.

range (0–8 μg/mL), as recommended for Comet assay by International Workshop on Genotoxicity Test Procedures [37].

DNA damage induction showed a dose–response relationship (Table 3) when measured as damage classes. Furthermore, all the treated samples are statistically different with respect to dose 0 for visual damage score, damaged cells, and by LDR (Table 4).

An increased cytotoxicity was observed when megazol and bleomycin were administered together (Fig. 2): at 2 μg/mL megazol cell viability is already less than 70%, deriving by both necrosis and apoptosis. In general,

Table 3
Megazol-induced cytotoxicity and DNA damage in rat leukocytes

	Cell survival (%)	DNA damage class frequency (%)			
		Class 1	Class 2	Class 3	Class 4
Dose (μg/mL)					
0	96.65 ± 0.47	80.33 ± 1.53	16.33 ± 1.53	3.33 ± 1.53	0
1	93.75 ± 0.99	51.00 ± 2.65	21.67 ± 3.06	10.67 ± 1.53	16.67 ± 1.53
2	89.08 ± 1.36	21.67 ± 2.52	43.00 ± 3.46	14.00 ± 1.73	21.33 ± 2.31
4	82.93 ± 5.03	7.33 ± 1.53	42.33 ± 3.79	15.33 ± 2.31	35.00 ± 1.00
8	76.87 ± 4.76	2.00 ± 1.00	18.33 ± 2.08	19.33 ± 2.08	60.33 ± 2.89
Bleomycin (μg/mL)					
50	95.38 ± 0.99	33.67 ± 2.08	25.33 ± 2.31	12.33 ± 2.08	28.67 ± 1.53
100	93.15 ± 2.43	4.67 ± 2.52	34.67 ± 3.06	9.00 ± 3.00	51.67 ± 0.58

DNA damage classes as defined in Section 2. Data obtained on cells from three animals.

Table 4
Genotoxic effects caused by megazol in rat leukocytes

	Damage score	Damaged cells	LDR
Dose (μg/mL)			
0	123 ± 3	19.7 ± 1.5	1.15 ± 0.04
1	193 ± 5***	49.0 ± 2.6***	1.77 ± 0.06***
2	235 ± 3***	78.3 ± 2.5***	2.93 ± 0.16***
4	278 ± 3***	92.7 ± 1.5***	3.93 ± 0.28***
8	338 ± 5***	98.0 ± 1.0***	8.03 ± 0.28***
Bleomycin (μg/mL)			
50	236 ± 6	66.3 ± 2.1	2.54 ± 0.14
100	307 ± 6	95.3 ± 2.5	7.39 ± 0.25

Damage score, damaged cells and mean LDR as described in Section 2. Mean ± SD of data obtained on cells of three animals are reported.
*** $P < 0.001$ (Kolmogorov–Smirnov test).

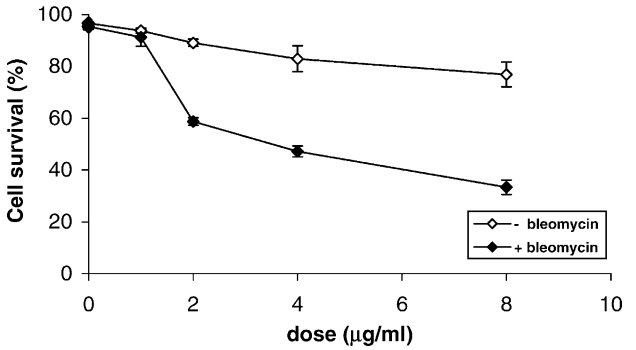


Fig. 2. Megazol toxicity in rat leukocytes, with (+) or without (−) 50 μg/mL bleomycin. The average survival and standard deviation for each tested dose are reported.

treatment of cultures with concentrations resulting in <70% relative viability were considered to be too cytotoxic and where not further evaluated; however, for evaluation of the influence of cytotoxicity on DNA migration, the first concentration exceeding this limit was analysed.

Dose–response graphs of some statistical parameters (mean, median, 95, and 99th percentile) of frequency distribution of DNA damage (LDR) were built (Fig. 3) for both experimental conditions (with or without bleomycin). A significant linear correlation was shown (for all LDR parameters: $R^2 > 0.84$). Furthermore, together with an increased DNA damage at increasing doses, a synergistic effect of megazol on bleomycin became evident: the overall effect was higher than the sum (theoretical values)

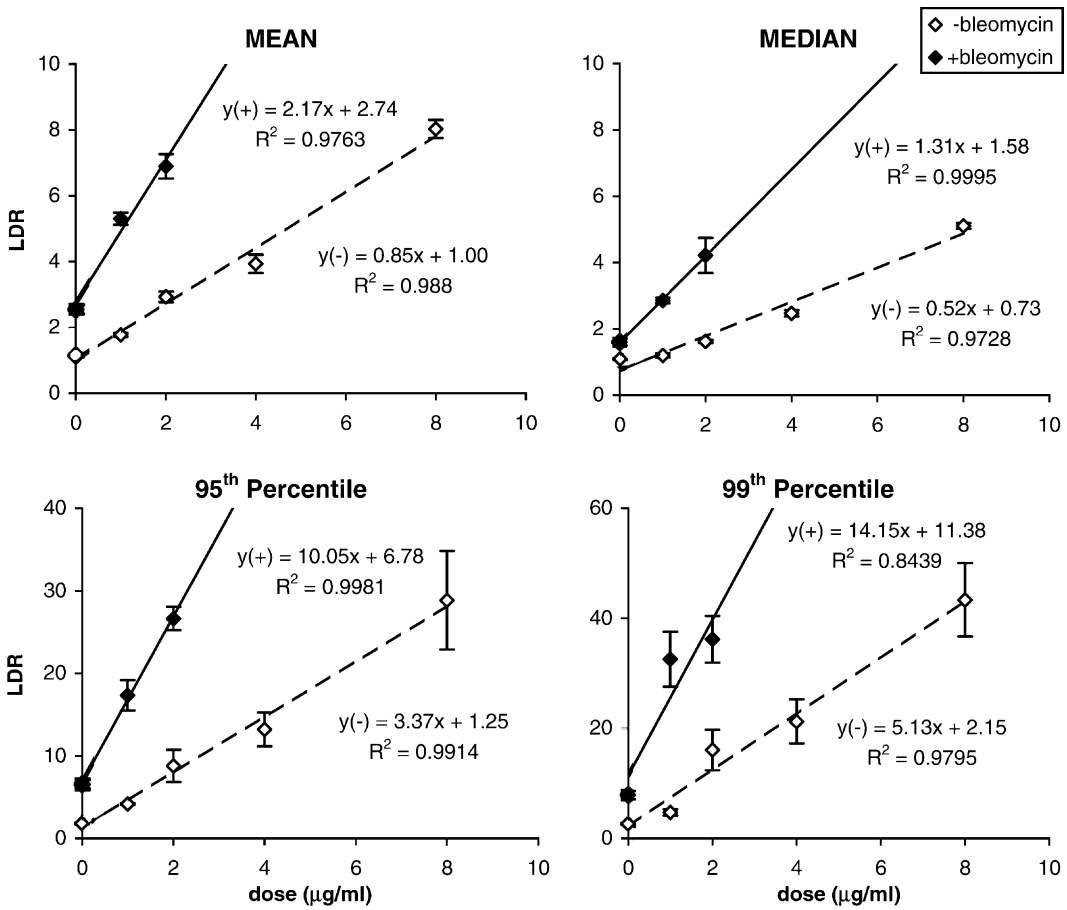


Fig. 3. Dose–response relationship of genotoxic effect in rat leukocytes after treatment with different doses of megazol in the absence (−) or presence (+) of bleomycin (50 μg/mL), in three independent experiments. Average mean, median, 95 and 99th percentile values (three independent experiments) of the Comet parameter LDR (length/diameter ratio) are shown. Regression lines are shown.

of individual effects for each xenobiotic (i.e. the sum of effect induced by 50 μg/mL bleomycin and different doses of megazol).

3.3. Mouse leukocytes

To expand the data obtained on rat cells to other mammalian cells, we also performed MZ treatment *in vitro* on fresh mouse leukocytes.

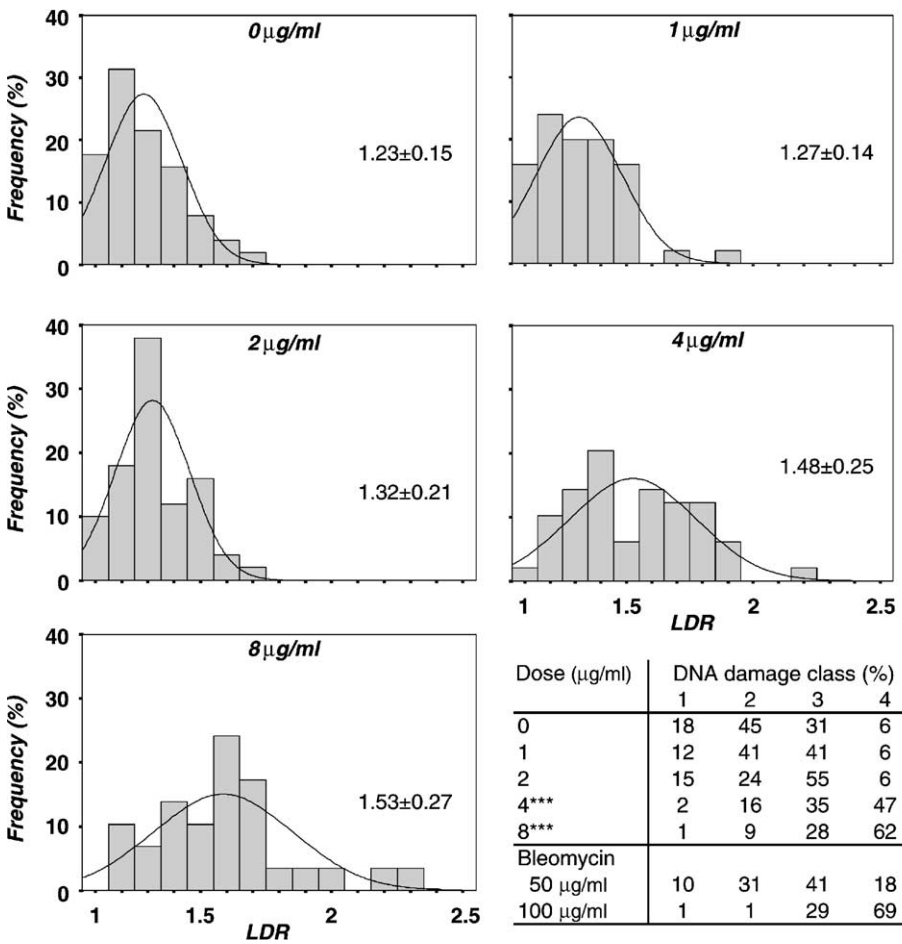
The findings obtained by the Comet assay also revealed a genotoxic action of drug in mouse cells (Fig. 4) and a dose–response DNA damage induction was found. Megazol is considered therapeutic at 25 mM which corresponds to about 4 μg/mL. Our data indicated that 4 μg/mL was the first significantly active dose (Kolmogorov–Smirnov test, $P < 0.001$). The low presence (4%) of hedgehog cells (or ghost cells) at the highest doses suggested a very low incidence of apoptotic/necrotic events during the treatment time and, consequently, the detected DNA migration may be ascribed to a MZ-induced genotoxic action. Cell viability, measured immediately after treatment, is always ≥70% in the dose range used (0–8 μg/mL) and comparable with rat cell viability.

3.4. VERO cells

We measured MZ-induced DNA damage at different times of treatment to evaluate the relationship among DNA damage induction/recovery and time of treatment. The cells were treated with MZ at 4 μg/mL for 1, 2, 4, 20, and 40 hr to eventually distinguish short (1, 2, 4 hr) and long (20 and 40 hr) exposure time. Untreated (control) and DMSO-treated samples (MZ was administrated when dissolved in DMSO) are considered. The data are reported as frequency distribution of Comet length/diameter ratio (Fig. 5). The graphs showed an increase of LDR mean values together with a displacement to the right (i.e. higher DNA damage) of frequency classes in MZ-treated samples. Therefore, the drug is active on DNA of VERO cells (Kolmogorov–Smirnov test, $P < 0.001$).

The effects could be better described by frequency distribution parameters other than mean, and specially with non-normal distributions. Therefore, median and percentiles are considered (Table 5). All the parameters resulted as good descriptors of the effect.

Two significantly different groups of damage (Dunnet's C, $P < 0.001$) are evident: the "short time" group (1, 2, 4 hr)



Dose (μg/ml)	DNA damage class (%)			
	1	2	3	4
0	18	45	31	6
1	12	41	41	6
2	15	24	55	6
4***	2	16	35	47
8***	1	9	28	62
Bleomycin				
50 μg/ml	10	31	41	18
100 μg/ml	1	1	29	69

Fig. 4. Frequency distribution of DNA damage in mouse leukocytes after treatment with megazol (data of one experiment). LDR = length/diameter ratio as defined in Section 2 (AU: arbitrary units). Mean ± SD are reported. DNA damage classes are reported both for megazol and positive controls (bleomycin) treatment. (***) $P < 0.001$ (Kolmogorov–Smirnov test).

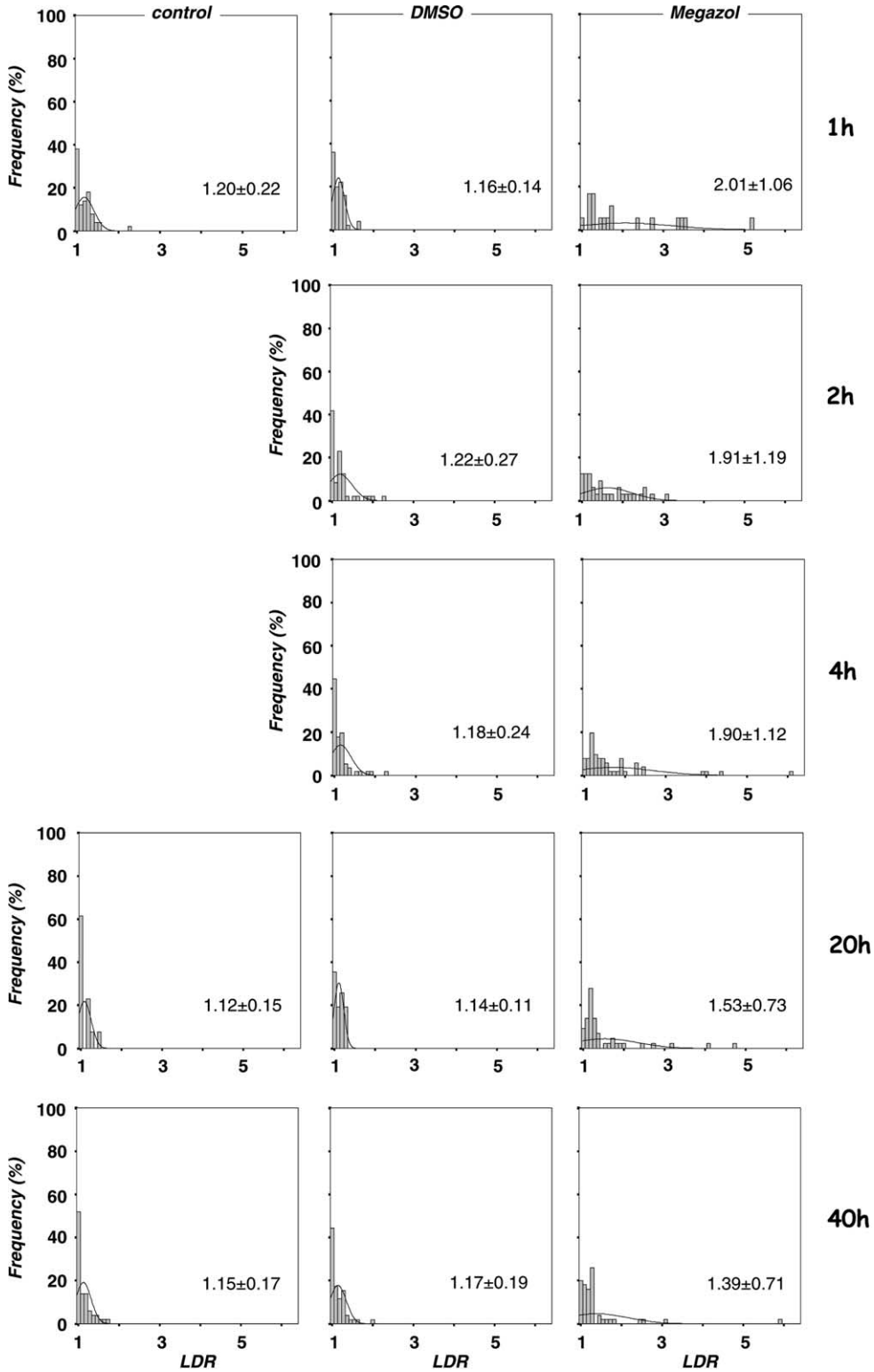


Fig. 5. Frequency distribution of DNA damage in VERO cells after treatment with megazol during different time. LDR = length/diameter ratio as defined in Section 2. Mean ± SD are reported.

showed a wider DNA damage than the ''long time'' group (20, 40 hr) suggesting a recovery process. Two possible ways could be suggested:

- a wide and intensive process of DNA repair,
- a high programmed cell death (apoptosis) and/or ''normal'' death (necrosis).

On the other hand, the survival rate of cells treated for 1, 2, 3, 24, and 48 hr with 25 μM megazol does not show any

Table 5
VERO cells exposed to megazol (4 μg/mL) at different time of treatment

Time (hr)	Treatment	Mean ± SD	Median	95th percentile	99th percentile
1	Control	1.20 ± 0.22	1.17	1.55	2.22
	DMSO	1.16 ± 0.14	1.15	1.45	1.65
	Megazol***	2.01 ± 1.04	1.59	4.31	4.88
2	DMSO	1.22 ± 0.27	1.17	1.93	2.22
	Megazol***	1.91 ± 1.19	1.46	5.51	6.00
4	DMSO	1.18 ± 0.24	1.14	1.78	2.25
	Megazol***	1.90 ± 1.12	1.40	4.57	5.71
20	Control	1.12 ± 0.15	1.04	1.46	1.50
	DMSO	1.14 ± 0.11	1.15	1.30	1.31
	Megazol**	1.53 ± 0.73	1.25	3.73	4.44
40	Control	1.15 ± 0.17	1.07	1.55	1.71
	DMSO	1.17 ± 0.19	1.14	1.57	2.00
	Megazol*	1.39 ± 0.71	1.20	2.68	5.57

Different DNA damage parameters (mean ± SD, median, and percentiles) of Comet LDR values (arbitrary units) are reported.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ (Kolmogorov–Smirnov test).

difference with respect to control (DMSO) cells (data not reported).

4. Discussion

The chemotherapy of Chagas' disease is still an open field and relies on few drugs which show toxicity and adverse side effects. Progress towards the development of novel therapeutics can be obtained from target identification and validation by chemical and genetic means through to rational drug design.

On the basis of a better understanding of the mode of action of the nitroimidazole derivative megazol, more active and safer drugs might be developed. Although the mode of action of this compound is not absolutely clear, it is generally assumed that its anti-microbial effect can be linked to the nitro group. As for other nitroheterocyclic drugs, the therapeutic effect of MZ appears linked with the chemical or enzymatic reduction of the nitro group: the single electron reduction of the compound by rat liver microsomes was confirmed [21]. On the other hand, in a previous report [19] the MZ nitro anion radical was not detected in the presence of liver microsome fraction. Furthermore, no significant increase in the number of induced mutants was detected in *Salmonella* strains in the presence of rat S9 [22,23].

Our findings on *S. cerevisiae* D7 strain did not show a significant increase of mutation and/or recombination event frequencies after treatment with megazol, both with and without endogenous activation by P450. On the other hand, a cytotoxicity was evident in cultures in the absence of P450 activity. In this case, the cytochrome complex appears to detoxify the drug. The induction of cytotoxicity, but no genotoxicity, suggests two hypothesis:

- megazol is unable to interact with DNA and its action is only at level of cellular membrane;
- the cellular wall, present in the yeast, protects the cell inhibiting drug uptake.

The effects of megazol on *Salmonella* [22,23] is in line with the second hypothesis. However, other nitroimidazoles were shown to be ineffective on DNA of eukaryotic systems [11,14–16]. Furthermore, it is known that the sensitivity of *S. cerevisiae* can be decreased by cell ability to transform some compounds, for example PAHs, in metabolites which are less mutagenic than the original compounds [38]. We treated different mammalian cells with megazol to assess DNA damage by the Comet assay.

Cells from two rodents (rat and mouse) were considered to assess species-specific sensitivity. Megazol was able to induce DNA damage in both cellular types although rat leukocytes showed a higher sensitivity. VERO cells was utilised as a model to evaluate the effects in relationship to treatment time: "short time" group (1, 2, 4 hr) showed a wider DNA damage than the "long time" group (20, 40 hr) suggesting a recovery process.

An increased cytotoxicity was observed when megazol and bleomycin were given together: at 2 μg/mL megazol cell viability is already less than 70% deriving by both necrosis and apoptosis. In general, treatment of cultures with concentrations resulting in <70% relative viability were considered to be too cytotoxic and where not evaluated; however, to examine the influence of cytotoxicity on DNA migration, the first concentration exceeding this limit was analysed. Really, it has yet not well been established how cytotoxicity influences DNA migration in the Comet assay [39–41]. Dead or dying cells can undergo rapid DNA fragmentation which should be

expected to increase DNA migration in the assay. On the other hand, the Comet assay permits the measurements on individual cells and therefore provides the advantage over other strand breaks assay such as alkaline elution, alkaline unwinding, sucrose-gradient since dead cells (both apoptotic and necrotic cells) can be identified by their distinct morphology as compared to cells exhibiting DNA damage. The morphology results from fragmentation of chromatin in such a way that cells with non-detectable Comet head are visible in the gels.

Our results strongly support the notion that extensive DNA fragmentation induced by cytotoxicity does not lead to elevated DNA migration (i.e. false positive cells) when ghost cells are not considered in genotoxicity evaluation. The potential problem of possible false positive cells due to excessive cytotoxicity arises from extensive DNA fragmentation upon cell death. Furthermore, the ghost cell score could provide a valuable parameter to obtain further information about cytotoxicity.

However, different cells, or cell lines, may behave differently and it was reported that non-genotoxins at cytotoxic concentrations may induce DNA migration in the Comet assay [42,43]. Therefore, we support the recommendation of the International Workshop on Genotoxicity Test Procedures [37] to limit concentrations of test compounds to $\geq$70% viability.

Megazol co-cytotoxic power is clearly shown. Co-genotoxicity is also demonstrated even if only two doses are considered on account of the high-induced cytotoxicity. However, other drugs/xenobiotics, with mechanisms and genetic end-points different from bleomycin, have to be tested to further clarify and confirm megazol co-genotoxic capability.

Megazol shows different biological activity in relation to cellular types (yeast and mammalian cells) and experimental conditions (with or without P450, short/long time of exposure, with or without other genotoxins). This suggests a possible modulation of drug effectiveness by different physiological/biochemical conditions of cells.

The present results suggest that the effects observed should warn against the potential risk of MZ treatment even if DNA repair systems could be induced. In this respect, we are specifically performing further studies on the mechanisms which are able to repair DNA damage induced by the drug. Furthermore, the findings on megazol could be useful to compare new megazol derived compounds and to assess the risks/benefits ratio for each drug.

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