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# RESTRICTED BIOREDUCTIVE METABOLISM OF A NITROIMIDAZOLE–THIADIAZOLE DERIVATIVE WITH CURATIVE ACTION IN EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTIONS

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**Abstract**—The bioreductive activation of megazol [2-amino-5(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole] promoted by ferredoxin: $NADP^+$ oxidoreductase, rat liver microsomes and cellular fractions of *Trypanosoma cruzi*, Y strain, was investigated. Direct ESR detection and characterization by computer simulation of the megazol nitro anion radical were possible in the presence of NADPH and ferredoxin: $NADP^+$ oxidoreductase under anaerobic conditions. By contrast, the megazol nitro anion radical was not detected in the presence of either rat liver microsomes or cellular fractions of *T. cruzi* under conditions where the corresponding nifurtimox anion radical was observed. The inefficiency of rat liver microsomes in catalyzing megazol reduction was also attested by visible light absorption spectroscopy. In the presence of cellular fractions of *T. cruzi* supplemented with NAD(P)H, megazol marginally affected oxygen consumption and decreased the yield of oxyradicals that can be spin-trapped with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Our results indicate a restricted bioreductive metabolism of megazol and suggest that its trypanocidal activity is unrelated to a redox-cycling process.

Megazol‡ [2-amino-5(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole] is a broad spectrum anti-bacterial and anti-parasitic compound [1–4]. The drug has also shown a marked curative effect in experimental Chagas' disease [5], being effective against strains that are resistant to the clinically employed agents nifurtimox and benznidazole [5, 6].

Megazol, nifurtimox and benznidazole are nitroheterocyclic compounds (structures shown in Fig. 1), a class of drugs widely used not only as antimicrobial agents but also in cancer therapy [7–10]. In general, the first step in the metabolism of these compounds is their reduction to the corresponding nitro anion radical [7–9]. Under anaerobic conditions, these radicals can undergo further reduction yielding nitroso, hydroxylamine and amine derivatives [7–9]. Under air, the nitro anion radical regenerates the parent nitro compound by reducing oxygen to the superoxide anion [7–9]; as a consequence, hydroxyl radicals can be generated from superoxide anion and $H_2O_2$ through Fenton-like chemistry [11]. The latter redox-cycling process has been implicated in the trypanocidal activity of nifurtimox, and is also considered a detoxification route for benznidazole [12, 13]. In contrast to nifurtimox and benznidazole, little is known about megazol metabolism. Considering its marked trypanocidal activity [5, 6], it is important to study the possibility of megazol redox-cycling catalyzed by *Trypanosoma cruzi* extracts. In this report we have characterized the megazol nitro anion radical and compared the reductive metabolism of megazol and nifurtimox by cellular fractions of epimastigote forms of *T. cruzi*, Y strain, and by rat liver microsomes.

## MATERIALS AND METHODS

NADH, NADPH, ferredoxin: $NADP^+$ oxidoreductase (EC 1.18.1.2, from spinach leaves), DTPA and DMPO were obtained from the Sigma Chemical Co. (St Louis, MO). DMPO was purified by charcoal filtration [14]. Nifurtimox was a gift from Bayer A.G. (Leverkusen, F.R.G.), benznidazole was from Hoffmann La Roche & Co. (Basel, Switzerland), and megazol was synthesized and supplied by Drs M. E. Gilbert and B. Gilbert. Stock solutions of both drugs were prepared in DMSO.

Epimastigote forms of *T. cruzi*, Y strain [15], were grown in liver Infusion-Tryptose (LIT) medium [16] supplemented with 10% bovine serum. The cells were collected by centrifugation and washed twice with phosphate buffered saline. The cells were then broken by freeze–thawing, and the two cellular fractions used, the 480 *g* supernatant fraction and the mitochondrial fraction, were obtained by centrifugation as previously described [17]. Rat liver microsomes were obtained by standard procedures [18]. Protein concentrations were determined by the biuret method [19] using serum albumin as standard.

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‡ Abbreviations and trivial names: megazol, 2-amino-5(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole; nifurtimox,3-methyl-4-(5′-nitrofurfurylidene-amino)- tetrahydro-4*H*-1,4-thiazine-1,1-dioxide; benznidazole, *N*-benzyl-2-nitro-1-imidazolacetamide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylene triaminepentaacetic acid; and DMSO, dimethyl sulfoxide.



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Oxygen consumption was measured polarographically with an oxygen monitor (Gilson 5/6 Oxygraph) at 37°; the saturating oxygen concentration at this temperature was taken as 0.20 mM [20]. The standard reaction mixture, final volume 1.8 ml, contained cellular fractions (4 mg protein/ml), NAD(P)H (1 mM), nifurtimox or megazol (1 mM), DMSO (10%, v/v), KCl (150 mM), DTPA (1.0 mM) and phosphate buffer (20 mM), pH 7.4. Additional components or changes in the incubation mixtures are given in the figure legends.

ESR spectra were recorded at room temperature on a Varian E-4 or on a Bruker ER-200D spectrometer. ESR spectra were obtained from aliquots transferred from incubation mixtures at 37° to gas permeable tubing (internal diameter 0.8 mm, wall thickness 0.05 mm, Zeus Industrial Products, Inc., Raritan, NJ, (U.S.A); this arrangement permits the recording of the ESR spectra under a controlled atmosphere [21]. The standard reaction mixture, final volume 0.1 ml, was the same as that for oxygen consumption studies except for the presence of 100 mM DMPO. ESR spectra simulations were carried out on a Bruker Aspect 2000 data acquisition system.

Optical absorption spectra were recorded on a Varian DMS 70 spectrophotometer at 37°, in 0.5-cm optical length cuvettes. The disappearance of the nitro chromophore was followed at 400 nm for both nifurtimox and megazol; this wavelength corresponds to the nifurtimox absorption maximum but it was also used for megazol (spectrum shown in Fig. 7, inset) to avoid interferences due to the pyridine nucleotides. The percentage of drug reduction was calculated by using values of $\Delta\varepsilon$ at 400 nm of 14.7/mM/cm and 3.8/mM/cm for nifurtimox and megazol respectively. These values were calculated from the differences in the absorbances at 400 nm of the parent drugs and the drugs reduced by sodium dithionite (see, for instance, Fig. 7, inset). The standard reaction mixture containing cellular fractions (2 mg protein/ml), nifurtimox or megazol (0.2 mM), DMSO (0.04%, v/v) KCl (150 mM), DTPA (1.0 mM) and phosphate buffer (20 mM), pH 7.4, was placed in a cuvette (0.5 cm optical length) containing a magnetic stirrer. The cuvette was capped with a rubber septum and nitrogen was bubbled through a needle for 15 min under stirring; after temperature equilibration at 37°, the reaction was started by addition of NAD(P)H (0.5 mM final concentration) through a microsyringe.

## RESULTS

*Megazol nitro anion radical.* Several heterocyclic and aromatic nitrocompounds have been shown to be chemically and enzymatically reduced to their nitro anion radicals [7–9]. However, to the best of our knowledge, the corresponding nitro anion radical of megazol has not been reported. In this context, we have started to study the bioreductive metabolism of megazol by characterizing its anion radical. A convenient model for enzymatic reduction of xenobiotics is the NADPH/ferredoxin: $NADP^+$-oxidoreductase system [11, 22, 23] and, indeed, incubation of 1 mM megazol with 1 mM NADPH and 0.04 units/ml of ferredoxin: $NADP^+$ oxidoreductase led to the appearance of the ESR spectrum displayed in Fig. 2A. This spectrum was ascribed to the megazol nitro anion radical anion based on its oxygen sensitivity (Fig. 2B) [7–9] and the dominant triplet ($a^n = 14.15$) (Fig. 2A). Indeed, computer simulation (Fig. 2C) of the experimental ESR spectrum was achieved by the following hyperfine coupling parameters: 1N ($NO_2$) $a^n = 14.15$; 2N (1, 3) $a^n = 0.94$; 1H (4) $a^h = 3.5$; 3H ($CH_3$) $a^h = 1.8$ (Fig. 2). (ring positions shown in Fig. 1). These values are comparable to those reported for metronidazole [24] although the spectra displayed in Fig. 2 (Fig. 2, A and C) did not show the resolution of the two ring nitrogens (positions 1 and 3), reported to be unequivalent for metronidazole [24].

*Bioreductive metabolism of megazol.* A detailed comparison of the reductive metabolism of megazol and nifurtimox was performed by using cellular fractions of epimastigote forms of *T. cruzi*, Y strain.

The results obtained with nifurtimox were comparable to those reported in previous studies in which the reductive metabolism generated the corresponding nitro anion radical under anaerobic conditions (Fig. 3A) [25–28]. Increased oxygen consumption (Fig. 4a and c; Table 1) and generation of oxyradicals (Fig. 5) [25–28] were observed in the presence of oxygen. These species were demonstrated by spin-trapping experiments with DMPO in the presence of DMSO. Under these conditions, the ESR spectra of the DMPO-methyl radical adduct ($a^n = 16.4$; $a^h = 23.4$) [29] and traces of the DMPO-superoxide radical adduct (labeled external lines) were detected (Fig. 5B) [26]. Detection of the DMPO-methyl radical adduct was due to the fact that the generated hydroxyl radical reacts with DMSO leading to the methyl radical that reacts with the spin-trap [29]. DMSO was used as solvent for nifurtimox, but it could be substituted by acetonitrile; in this case the DMPO-hydroxyl radical adduct ($a^n = 14.9$; $a^h = 14.9$) [29] was directly observed (not shown). However, megazol is of low solubility in acetonitrile and, for comparative purposes, the experiments were carried out in the presence of DMSO used as the drug solvent (Materials and Methods).

By contrast to nifurtimox, megazol nitro anion radical was not detected in mitochondrial suspension of *T. cruzi* under anaerobic conditions (Fig. 3B). Under aerobic conditions, mitochondrial respiration was marginally affected by megazol (Fig. 4b; Table 1), and the yield of radicals that were spin-trapped with DMPO was lower than that obtained in the absence of the drug (Fig. 5). This result was not altered in the presence of cyanide although, as expected [30], this respiratory inhibitor increased the yield of oxyradicals in the presence or in the absence of nifurtimox (not shown).

Addition of megazol to the 480 *g* supernatant fractions of *T. cruzi* supplemented with NAD(P)H led to an increase in oxygen consumption immediately after drug addition, but the rate stabilized in a value close to the control (Fig. 4d; Table 1). Also, as observed in the presence of mitochondrial fractions (Fig. 5), megazol inhibited the yield of the spin-trapped radicals obtained in the presence of the 480 *g* supernatant fraction (Table 1). These results indicate

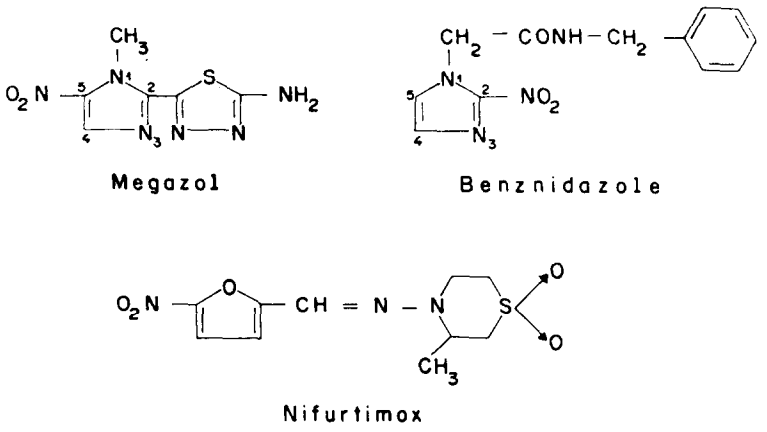


Fig. 1. Structures of megazol, benznidazole and nifurtimox.

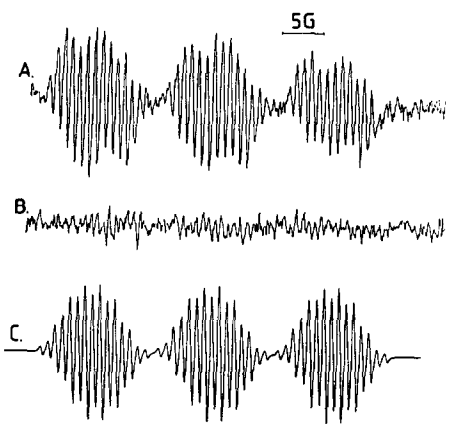


Fig. 2. ESR spectra obtained during megazol reduction. Megazol (1 mM) was incubated with ferredoxin:$NADP^+$ oxidoreductase (0.04 units/ml) and NADPH (1 mM) in phosphate buffer (20 mM), pH 7.4, containing DTPA (1 mM) and DMSO (10%, v/v) under: (A) anaerobic conditions, and (B) air. Computer simulation of (A) is shown in (C). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.5 G; time constant, 0.5 sec; scan rate, 0.25 G/sec.

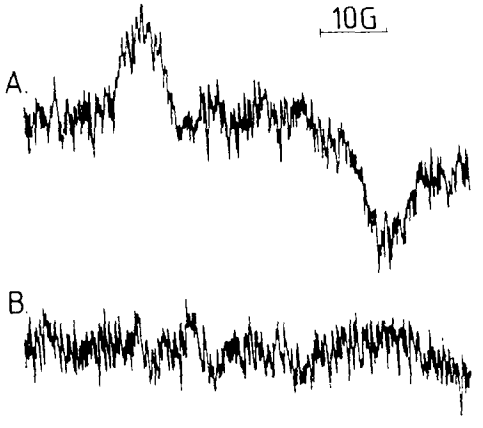


Fig. 3. ESR spectra obtained during anaerobic incubations of *Trypanosoma cruzi* mitochondrial fraction with nifurtimox or megazol. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, contained *T. cruzi* mitochondrial fraction (4 mg protein/ml), NADH (1 mM), DTPA (1 mM), KCl (150 mM), DMSO (10%, v/v) and: (A) nifurtimox (1 mM); and (B) megazol (1 mM). Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.5 sec; scan rate, 0.21 G/sec.

that the extra oxygen consumption observed upon addition of megazol (Fig. 4) cannot be attributed to redox-cycling of the drug. Indeed, there was no evidence for: (i) generation of the anion radical under anaerobic conditions (the results obtained with the 480 *g* supernatant fraction were similar to those shown in Fig. 3), or (ii) increased generation of oxyradicals in spin-trapping experiments (Table 1).

Nitro radical anions can be produced enzymatically by several enzymes such as ferredoxin: $NADP^+$ oxidoreductase, xanthine oxidase, aldehyde oxidase and NADPH:cytochrome $P_{-450}$ (cytochrome *c*) reductase [10, 22]. Considering that some of these enzymatic activities are still poorly characterized in *T. cruzi* [13, 31, 32], we compared the bioreductive activation of megazol and nifurtimox by rat liver microsomes supplemented with NADPH. Again, there was no evidence for generation of the megazol nitro radical anion under conditions where the corresponding nifurtimox- and benznidazole-derived free radicals could be observed (Fig. 6). Megazol nitro radical anion was not detected by direct ESR spectroscopy even when its concentration was increased twice; the use of much higher concentrations was precluded by the low solubility of megazol. Due to the qualitative characteristics of the ESR spectroscopy [33], reduction of megazol or nifurtimox by rat liver microsomes under anaerobic conditions was also followed by visible light absorption spectroscopy, as described in Materials and Methods. In 5 min, about 50% of nifurtimox was reduced whereas megazol remained little affected (Fig. 7), further attesting to the inefficiency of rat liver microsomes in reducing magazol. A similar comparison in the presence of cellular fractions of *T. cruzi* was difficult because the reduction rates were very slow leading to scattered results; for instance, less than 10% of nifurtimox was reduced

Table 1. Effects of nifurtimox and megazol upon oxygen consumption by *Trypanosoma cruzi* cellular fractions supplemented with NAD(P)H*

| Cellular fraction | Additions | Oxygen consumption†‡ (nmol $O_2$/ min/mg protein) | ESR signal height†§ (arbitrary units) |
|---|---|---|---|
| Miochondria | NADH | 1.5 | 1.4 |
| | NADH + nifurtimox | 2.6 | 4.2 |
| | NADH + megazol | 1.7 | 1.0 |
| 480 *g* Supernatant | NADH | 1.9 | 3.0 |
| | NADH + nifurtimox | 3.4 | 16.9 |
| | NADH + megazol | 2.3 | 0.8 |
| | NADPH | 1.0 | 1.6 |
| | NADPH + nifurtimox | 3.1 | 15.0 |
| | NADPH + megazol | 1.3 | 0.5 |

* Standard incubation mixtures containing *T. cruzi* cellular fractions (4 mg protein/ml), NAD(P)H (1 mM), DMSO (10%, v/v), nifurtimox or megazol (1 mM), KCl (150 mM), DTPA (1.0 mM) and phosphate buffer (0.1 M), pH 7.4. For the ESR experiments, DMPO (100 mM) was also added.

† Average of experiments with two different cellular fraction preparations.

‡ Rates measured in the linear portion of the curves; see, for instance, Fig. 4.

§ Peak height of the low field signal of the DMPO-methyl radical adduct after a 15-min incubation at 37°.

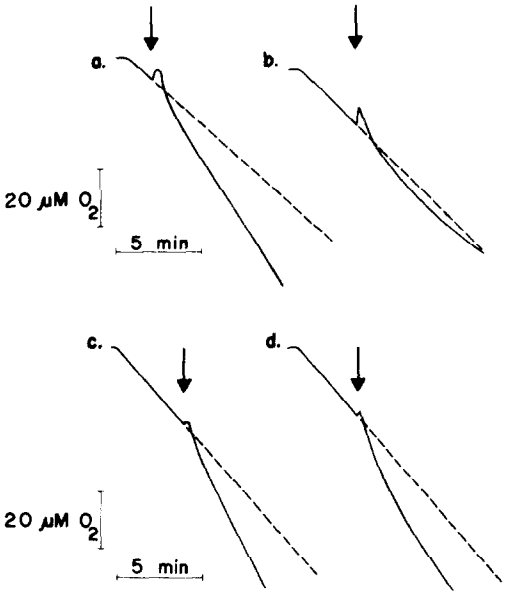


Fig. 4. Oxygen consumption by *Trypanosoma cruzi* cellular fractions supplemented with NADH. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, were incubated at 37° and contained *T. cruzi* cellular fractions (4 mg protein/ml), NADH (1 mM), DTPA (1 mM), KCl (150 mM) and DMSO (10%, v/v). At the points indicated by the arrows, the nitroheterocyclic compounds were added to 1 mM final concentration. Key: (a) mitochondrial fraction plus nifurtimox; (b) mitochondrial fraction plus megazol; (c) 480 *g* supernatant fraction plus nifurtimox; and (d) 480 *g* supernatant fraction plus megazol. The broken lines extrapolate the oxygen consumption in the absence of the drugs, as parallel experiments have demonstrated that it remains linear up to 15 min.

after a 1-hr incubation with *T. cruzi* 480 *g* supernatant fraction supplemented with NADPH (not shown).

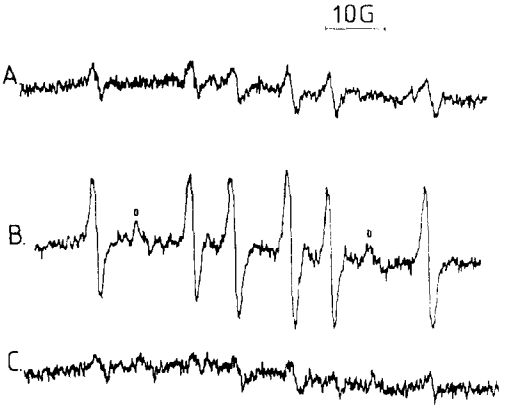


Fig. 5. ESR spectra of the DMPO-radical adducts obtained during incubations of *Trypanosoma cruzi* mitochondrial fraction with nifurtimox or megazol. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, were incubated for 15 min at 37° and contained *T. cruzi* mitochondrial fraction (4 mg protein/ml), DMPO (100 mM), DTPA (1 mM), KCl, (150 mM), DMSO (10%, v/v) and: (A) NADH (1 mM); (B) NADH (1 mM) plus nifurtimox (1 mM); and (C) NADH (1 mM) plus megazol (1 mM). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.5 sec; scan rate, 0.21 G/sec.

## DISCUSSION

Our studies have demonstrated that reductive activation of megazol generates the corresponding nitro radical anion but requires enzymes with low redox potential. In the presence of NADPH and ferredoxin: $NADP^+$ oxidoreductase ($E_m = -0.442$ mV) [34], the megazol nitro anion radical was easily detected by direct ESR spectroscopy under anaerobic conditions (Fig. 2A) and was well characterized by computer simulation (Fig. 2C) of the experimental spectrum (Fig. 2A) [24]. On the other hand, there was no indication for the generation of the megazol

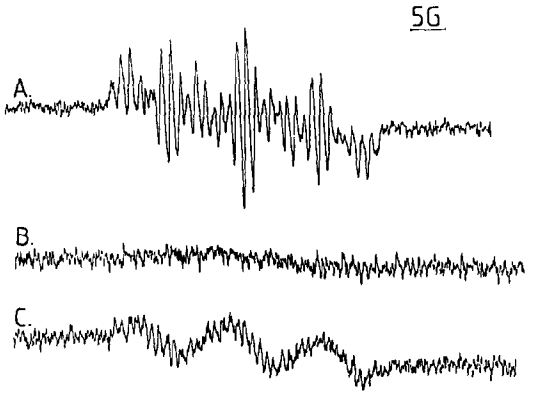


Fig. 6. ESR spectra obtained during anaerobic incubations of rat liver microsomes with the nitroheterocyclic compounds. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, contained rat liver microsomes (2 mg protein/ml), NADPH (5 mM), DTPA (1 mM), KCl (150 mM), DMSO (10%, v/v) and: (A) nifurtimox (5 mM); (B) megazol (10 mM); and (C) benznidazole (10 mM). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.3 sec; scan rate, 0.21 G/sec.

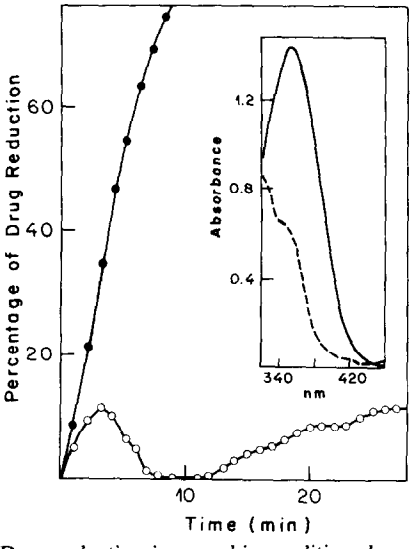


Fig. 7. Drug reduction in anerobic conditions by rat liver microsomes. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, were incubated at 37° and contained rat liver microsomes (2 mg protein/ml), NADPH (0.5 mM), DTPA (1 mM), KCl (150 mM), DMSO (0.04%, v/v) and: (●) nifurtimox (0.2 mM); and (○) megazol (0.2 mM). The inset displays the visible light absorption spectra of megazol (0.2 mM) in buffer before (——) and after (– – –) addition of sodium dithionite in both the blank and the sample cuvettes.

nitro anion radical in the presence of NAD(P)H/cellular fractions of *T. cruzi* Y strain (Fig. 3) or in the presence of NAD(P)H/rat liver microsomes (Fig. 6). Although *T. cruzi* nitroreductases are not well characterized [13, 31, 32], the results shown in Fig. 6 demonstrated that NADPH: cytochrome $P_{450}$ reductase ($E_m = -0.328$ mV) [35] is also inefficient in promoting the reduction of megazol to the nitro anion radical. Indeed, under these experimental conditions the megazol nitro anion radical was not detected, whereas the corresponding radicals of nifurtimox and benznidazole were detected (Fig. 6). The inefficiency of rat liver microsomes in reducing megazol was confirmed by experiments demonstrating the slow disappearance of the nitro chromophore as measured by visible light absorption spectroscopy (Fig. 7). Taken together, these results indicate a restricted bioreductive metabolism of megazol. In agreement, the known electron affinity of nitroheterocyclic compounds follows the order nitrofurans > 2-nitroimidazoles > 5-nitroimidazoles (Fig. 1); the parent nitroaryl structures have their reduction potential estimated as −330 mV, −390 mV and −470 mV respectively [7].

The resistance of megazol to reduction should be, at least partially, responsible for the fact that cellular fractions of *T. cruzi* did not catalyze redox-cycling of the drug. Although megazol marginally increased oxygen consumption by the 480 *g* supernatant fraction of *T. cruzi* (Fig. 4d, Table 1), this effect could not be ascribed to redox-cycling of the drug as the extra oxygen consumption did not lead to an increased generation of oxyradicals (Table 1). On the contrary, the yield of radicals spin-trapped by DMPO in the presence of megazol was always lower than in the control (Fig. 5; Table 1). This effect of megazol causing a transient increase of oxygen consumption and in inhibition of the yield of active oxygen species cannot be explained at the present time, but it is clearly different from the effect of nifurtimox [25–28]. Consequently, the marked trypanocidal activity of megazol [5, 6] should be independent of a redox-cycling process as previously suggested for benznidazole [36]. Our results indicate that bioreduction of megazol is less efficient than that of benznidazole however, suggesting that these drugs may have a different mechanism of action. Further studies will be required to clarify this point.

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