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Enzymatic Reduction Studies of Nitroheterocycles

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ABSTRACT. The nitroimidazole derivative Megazol is a highly active compound used against several strains of *Trypanosoma cruzi*, the causative agent of Chagas' disease (American trypanomiasis). With the aim of gaining an insight into the probable mode of action, the interaction of Megazol with different redox enzymes was studied in comparison to that of Nifurtimox and Metronidazole. The three nitroaromatic compounds are reduced by L-lactate cytochrome *c*-reductase, adrenodoxin reductase, and NADPH:cytochrome P-450 reductase (EC 1.6.2.4), the efficiencies of the enzymatic reductions being roughly related to the reduction potentials of these pseudo-substrates. As the enzyme responsible for the reduction of Megazol within the parasite has not yet been identified, the nitroimidazole was assayed with *T. cruzi* lipoamide dehydrogenase and trypanothione reductase. Megazol did not inhibit the physiological reactions but proved to be a weak substrate of both flavoenzymes. The single electron reduction of the compound by NADPH:cytochrome P-450 reductase, by rat liver as well as by trypanosome microsomes was confirmed by ESR experiments. As shown here, Megazol interferes with the oxygen metabolism of the parasite, but its extra activity when compared to Nifurtimox may be related to other features not yet identified. BIOCHEM PHARMACOL **57**;5:549–557, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nitroheterocycle; oxygen metabolism; nitro anion radical; trypanothione reductase; lipoamide dehydrogenase; Chagas' disease

Nifurtimox and Metronidazole (Fig. 1) are nitroheterocycles which exhibit, as do other nitrocompounds, antibacterial, antiprotozoal, and radiosensitizing activities [1–3]. These properties have been related to their electron affinity and more precisely to the reduction potential of the one-electron transfer $ArNO_2/ArNO_2^{\cdot-}$ [4, 5].

Megazol [1-methyl-2-(5-amino-1,3,4-thiadiazole)-5-nitroimidazole] is effective against *Trypanosoma cruzi*, the causative agent of Chagas' disease in South America. The compound is of particular interest since it is active against different strains of the parasite. Taking into account the resistance development and severe side effects of Nifurtimox and Benznidazole, the currently available drugs, Megazol represents a promising alternative. It has been shown that this compound is also highly active against *Trypanosoma brucei* in combination with Suramin [6] or Melarsoprol [7]. Although nitrocompounds are suspected of possible carcinogenicity [8], the high efficiency of Megazol may counterbalance this drawback in critical situations. On the basis of a better understanding of the mode of action of aromatic nitrocompounds and particularly Megazol, more active and safer drugs might be developed. Therefore, this compound is being extensively investigated based on the well-documented sensitivity of trypanosomes and particularly *T. cruzi* towards oxidative stress. They lack catalase and glutathione peroxidase, and GR** is replaced by a parasite-specific TR [9–12]. Therefore, compounds that are able to induce oxidative stress or inhibit one of the detoxification enzymes specific to the parasite are of interest.

In general, nitrocompounds are good candidates, through their corresponding radical anions, to interfere with oxygen metabolism. Figure 1 gives the two routes of possible reactions evidenced for Nifurtimox [13, 14]: (a) Under anaerobic conditions, the radical anion formed in the first step can be transformed into the corresponding nitroso derivative. This nitroso form has been put forward as an efficient scavenger of essential thiols in the cell [15], the latter then undergoing further reduction to the amine. (b) Under aerobic conditions, the nitro radical anion reacts

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** *Abbreviations:* DMPO, 5,5′-dimethyl-pyrroline-*N*-oxide; DTPA, diethyl triamine pentaacetic acid; LipDH, lipoamide dehydrogenase (EC 1.8.1.4); TR, trypanothione reductase (EC 1.6.4.8); GR, glutathione reductase (EC 1.6.4.2); ADX, adrenodoxin; ADR, adrenodoxin reductase (EC 1.18.1.2); K_{cat}, bimolecular rate constant; and Ki, unimolecular rate constant.

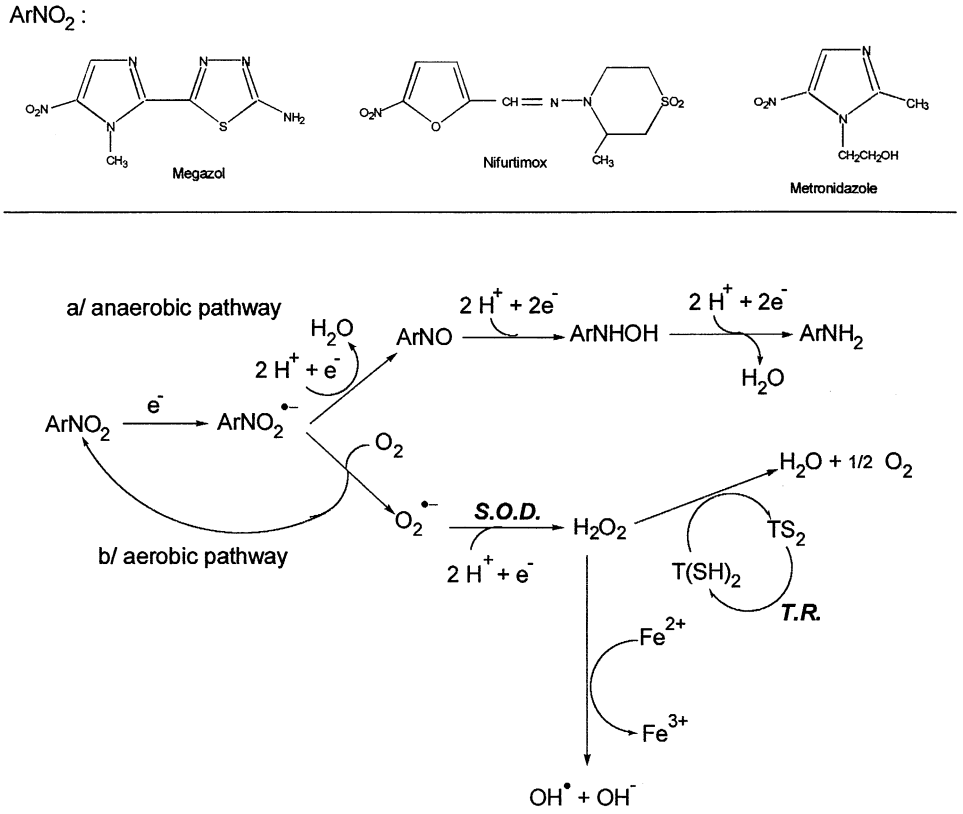

FIG. 1. Structure of Megazol, Nifurtimox, and Metronidazole and bioreductive pathway of aromatic nitrocompounds. SOD, superoxide dismutase.

with oxygen according to the futile cycle to form superoxide anions, which then are transformed into hydrogen peroxide by the reaction of superoxide dismutase. If hydrogen peroxide accumulates, it can generate $^{\cdot}$OH, a highly damaging species for the cell [13, 16].

The reductive metabolism was well demonstrated by ESR experiments for Nifurtimox [17, 18] and Metronidazole [19], but Tsuhako [20] indicated that the trypanocidal activity of Megazol was unrelated to a redox cycling process. The nitro radical anion could not be characterized by ESR under conditions used for Nifurtimox, namely by incubating rat hepatic microsomes or trypanosome extracts with the compound.

In this context, the probability of the reduction of Megazol should be addressed, particulary if the low reduction potential of this compound (−0.430 V) [21] is considered. The reaction of Megazol was therefore studied with three enzymes operating at largely different reduction potentials. The present study demonstrates by ESR experiments that Megazol can undergo bioreduction, yielding the corresponding nitro anion radical.

Since the nitroreductase that is responsible for the first reduction step of nitrocompounds used in Chagas' disease therapy has not yet been identified, Megazol was studied as a substrate of pig LipDH, an enzyme of known nitroreductase activity [22], and present in *T. cruzi* [23, 24]. In addition, the mode of action of Megazol was also investigated by assays on yeast and human GR, as well as *T. cruzi* TR. The present study also complements the pulse radiolysis work on Megazol, Nifurtimox, and Metronidazole [21].

MATERIALS AND METHODS

Materials

NADPH, NADH, DMPO, DTPA, and GR from yeast (Grade III) were obtained from Sigma, and cytochrome b_2 (L-lactate:cytochrome *c* reductase-EC 1.1.2.3) of *Hansenula anomala-* from Fermentas Scientific Corporation. NADPH:ADR and ADX from bovine adrenal cortex mitochondria were a generous gift from Professor S.A. Usanov (Institute of Bio-organic Chemistry, Minsk, Belorussia). Rat liver microsomes were kindly provided by Dr. M. Delaforge (University René Descartes, Paris). NADPH:cytochrome P-450 reductase from rat liver was purified by Dr. Benoît (University Claude Bernard, Lyon, France). Protein concentrations were determined by the Bradford method [25]. Pig heart lipoamide dehydrogenase (diaphorase) was purchased from Boehringer. Recombinant *T. cruzi* TR [26], LipDH [24], and human GR [27] were prepared as described. Lipoamide disulfide was obtained from Serva. Dihydrolipoamide was prepared by $NaBH_4$ reduction according to published procedures [28]; trypanothione TS_2, was purchased from Bachem. The enzyme concentrations were determined using $\epsilon_{460} = 11.3\ mM^{-1}\ cm^{-1}$ (GR, ADR), $\epsilon_{460} = 22\ mM^{-1}\ cm^{-1}$ (cytochrome P-450 reductase), $\epsilon_{414} = 10\ mM^{-1}\ cm^{-1}$ (ADX), $\epsilon_{423} = 183\ mM^{-1}\ cm^{-1}$ (cytochrome b_2, reduced by 10 mM L-lactate) [29]. Cytochrome *c* and L-lactate were obtained from Serva. Metronidazole was obtained from Polfa and Nifurtimox from Bayer A.G. Megazol, [1-methyl-2-(5-amino-1,3,4-thiadiazole)-5-nitroimidazole], first prepared by

American Cyanamid [30], was resynthesized according to improved procedures [31] and recrystallized twice from acetone. *T. cruzi* microsomes were obtained by centrifugation from *T. cruzi* epimastigotes (Sao Paulo strain) as previously described [32].

Interaction of Megazol and Nifurtimox with ADR, cytochrome P-450 reductase and cytochrome b_2

All experiments were performed in 0.1 M potassium phosphate buffer (pH 7.0, 1 mM EDTA) at 25°. The activities of the enzymes are expressed as k_{cat}. The kinetic constant k_{cat} of NADPH:cytochrome P-450 reductase, determined as the rate of enzymatic reduction of 50 μM cytochrome *c* by 50 μM NADPH using $\epsilon_{550} = 20\ mM^{-1}\ cm^{-1}$, was 30 sec^{-1}. The cytochrome *c* reductase activity of ADR in the presence of NADPH and 0.4 μM ADX was 10 sec^{-1}. Cytochrome b_2 activity determined by the enzymatic reduction of 1 mM ferricyanide ($\epsilon_{420} = 1\ mM^{-1}\ cm^{-1}$) by 10 mM L-lactate was 1000 sec^{-1}.

The bimolecular rate constants (k_{cat}/K_m) for the reduction of the nitrocompounds were determined from reciprocal slopes of Lineweaver–Burk plots (coordinates [E]/v, 1/[nitrocompound]). Typically, the oxidation of 30–40 μM NADPH was monitored in the presence of catalytic amounts of NADPH:cytochrome P-450 reductase or ADR. In the latter case, the reaction mixture also contained 0.4 μM ADX. The spectrophotometric measurements were performed at 340 nm using a Hitachi MPF-4 spectrophotometer. Due to the high absorbance of Megazol at 340 nm, the kinetics of NADPH oxidation were monitored spectrofluorimetrically (excitation 340 nm, emission 440 nm) using a Hitachi MPF-4 spectrofluorimeter. The rate of reduction of nitrocompounds by cytochrome b_2 in the presence of 10 mM L-lactate was monitored by an oxygen electrode following the rate of oxygen consumption due to the reoxidation of the formed nitro anion radicals.

ESR Experiments

ESR spectra were recorded at 37° on a Bruker ESR-600 spectrometer equipped with a temperature regulator system. The standard reaction mixture contained rat liver or *T. cruzi* microsomes, or NADPH:cytochrome P-450 reductase at concentrations indicated in the figures, and 1 mM DTPA. The nitroheterocycles were dissolved in DMSO or acetonitrile 10% v/v of the final concentration (at the concentration indicated in the figure), phosphate buffer (20 mM), KCl (150 mM), pH 7.4. The reaction mixture was transferred in 50 μL capillary immediately before the reaction was started by addition of NADPH (1 mM, final concentration).

The reaction mixture for ESR spectra of DMPO radical adducts was the same except for the presence of 100 mM DMPO. ESR spectra of the nitro anion radical were obtained from aliquots where argon was bubbled through a needle with a microsyringe for two min before the addition of NADPH; the reaction mixture was transferred into a 50 μL capillary, then sealed at both ends.

Interaction of Megazol and Nifurtimox with GR

Yeast GR activity, determined according to the rate of oxidation of 50 μM NADPH ($\epsilon_{340} = 6.2\ mM^{-1}\ cm^{-1}$) by 300 μM glutathione disulfide, was 220 sec^{-1}. The inhibition constants of yeast GR were determined either at various concentrations of NADPH (10–50 μM), or at various concentrations of glutathione (50–300 μM) and calculated according to Dixon plots [32]. All experiments were performed in 0.1 M potassium phosphate, pH 7.0 containing 1 mM EDTA, at 25°.

Single-electron reduction of Megazol and Nifurtimox by human GR was measured as described for TR. The reaction mixture (90 μL) contained 100 μM NADPH, 50 μM cytochrome *c*, 20 μg GR, and 0.3–1 mM nitrocompound in 50 mM potassium phosphate, pH 7.5.

Interaction of Megazol and Nifurtimox with TR

The *T. cruzi* TR activity was determined as previously described [33] using trypanothione as a disulfide substrate. Inhibition of TR by Megazol was studied in 1 mL assay mixtures containing 100 μM NADPH, 50 or 100 μM TS_2, and 5 mU TR in 50 mM Hepes, 1 mM EDTA, pH 7.5 at 25°, and 10–100 μM Megazol. The absorption decrease at 340 nm due to NADPH consumption was measured.

One-electron reduction of Megazol and Nifurtimox by TR was followed by the absorption increase at 550 nm ($\epsilon = 20\ mM^{-1}\ cm^{-1}$) due to formation of reduced cytochrome *c*. The reaction mixture (1 mL) contained 100 μM NADPH, 16 μM cytochrome *c*, 600 mU TR and 50 or 100 μM of the nitrocompound in 50 mM Hepes, 1 mM EDTA, pH 7.5. The activity was corrected for cytochrome *c* reductase activity of TR measured in the absence of any nitrocompound [34].

Interaction of Megazol and Nifurtimox with LipDH

The activity of pig heart and *T. cruzi* LipDH was measured both in the forward and the reverse reaction. For the forward reaction, the standard assay mixture contained (in 1 mL total volume) 1 mM dihydrolipoamide, 0.6 mM NAD, and 0.1–1.0 μg LipDH in 50 mM potassium phosphate, 1 mM EDTA, pH 8.0. The reaction was started by addition of the enzyme. The absorption increase at 340 nm at 25° was monitored. In the reverse reaction, a 1 mL assay mixture contained 1 mM lipoamide (disulfide) and 0.25 mM NADH in 50 mM potassium phosphate, 1 mM EDTA, pH 7.0. The influence of Megazol on the activity of LipDH was studied in the reverse reaction by adding 100 μM Megazol to the assay mixture containing 100 μM NADH and 120 μM lipoamide; in the forward reaction, the dihydrolipoamide concentration was 100 μM.

The one-electron reduction of the nitrocompounds by

TABLE I. Enzymatic reduction of nitrocompounds by different reductases

Nitrocompounds E_1^7 (mV) [ref. 20]	Megazol −438	Nifurtimox −260	Metronidazole −485
Enzymes	K_{cat}/K_m [M^{-1} sec^{-1}]		
L-lactate cyt. *c* reductase	4.7×10^3	2.8×10^3	ND
ADR	3.0×10^3	5.0×10^4	3.0×10^2
Cyt. P-450 reductase	2.0×10^3	2.0×10^4	ND
T. cruzi LipDH	2.3×10^2	7.6×10^2	ND
Pig heart LipDH	4.3×10^1	3.0×10^2	ND
T. cruzi TR	1.8×10^2	1.5×10^3	ND
Human GR	≤5	≤14	ND

The kinetics were measured at 25° as described under Materials and Methods. The activities of ADR and cytochrome P-450 reductase represent the rate of NADPH oxidation. The activity of L-lactate cytochrome *c* reductase was determined by measuring O_2 consumption. The k_{cat} values of the other enzymes were obtained from the coupled cytochrome *c* assays. The catalytic effiencies were derived from reciprocal slopes of Lineweaver–Burk plots ([E]./*v* = f(1/[nitro-compound]). ND = not determined.

LipDH was measured as described for TR. The assay mixture (1 mL) contained 100 μM NADH, 50 μM cytochrome *c*, and 20 μg LipDH in 50 mM potassium phosphate, pH 7.5, in the absence and presence of 100–1000 μM Megazol and 50–100 μM Nifurtimox, respectively.

RESULTS

Reduction of Megazol and Nifurtimox by Cytochrome b_2, ADR, and cytochrome P-450 reductase

Reductions of the three nitroaromatic compounds Megazol, Nifurtimox, and Metronidazole were studied using cytochrome b_2, ADR, and cytochrome P-450 reductase as reducing enzymes.

As described in the experimental section, the activity of cytochrome b_2 was measured by monitoring oxygen consumption. The reaction of the other two enzymes was determined by observing the oxidation of NADPH. The reduction route is similar to those for other reductases, particularly cytochrome P-450 reductase [35, 36]. The reduction of the nitroderivative is pulled by oxygen consumption and production of hydrogen peroxide.

The data presented in Table I reflect the rate of aerobic single-electron reduction of the nitrocompound; this reduction was followed by the formation of oxygen radicals by the same enzyme, demonstrating that such oxygen radicals have no effect on the enzyme activity [37]. The results indicate that the three enzymes catalyze the reduction of the nitrocompound: with cytochrome b_2, reduction of Nifurtimox and Megazol occurred at a similar rate, whereas with cytochrome P-450 reductase, reaction with the nitrofuran was faster by one order of magnitude. With ADR as well, the reaction was faster with Nifurtimox than with Megazol. The three values available for this enzyme indicate that catalytic efficiencies correlate with the reduction potentials of the nitrocompounds. These results show that Megazol is reduced by the three enzymes, and that there is no correlation between the rates of formation of the corresponding radical anions and the biological activity of these compounds [38, 39].

ESR Evidence for Enzymatic Production of Megazol Radical Anion

Using the radical anion described for Nifurtimox as a reference, parallel experiments were carried out with this compound and Megazol under anaerobic conditions. For the two compounds, one-electron reduction products were obtained (Fig. 2), both with the purified NADPH: cytochrome P-450 reductase (data not shown) and rat liver microsomes; no signal was observed in the absence of the biological extract or NADPH cofactor. The corresponding spectra are in agreement with those already published for Nifurtimox [17] and those obtained for Megazol after reduction by ferredoxin:$NADP^+$ oxidoreductase [20]. The signal obtained with Megazol, although of lower intensity under the same conditions, was observed to be longer than that for Nifurtimox. Half-lives determined under the same conditions were 10 and 3 min, respectively.

For experiments performed under aerobic conditions, the superoxide anion resulting from the fast reaction of oxygen with the nitro anion radical was trapped with DMPO. Similar spectra (Fig. 3) were obtained with NADPH:cytochrome P-450 reductase for both compounds, and these clearly correspond to the spectrum of the DMPO–$O_2^{\cdot -}$ adduct already described in detail [40, 41]. After a longer incubation time, the spectra corresponding to the DMPO–radical adducts became more complex, but in fact the extra signals correspond to the DMPO–$HO^{\cdot}$ adduct resulting from the former, as confirmed in the spectrum recorded after 10-min incubation. It has indeed previously been shown that the DMPO–$O_2^{\cdot -}$ complex converts into the DMPO–$HO^{\cdot}$ complex [42, 43].

When we performed the same experiments in DMSO instead of acetonitrile, the DMPO–$CH_3^{\cdot}$ radical was obtained as a final signal; this signal results from the trapping by DMPO of the methyl radical, resulting in its turn from $HO^{\cdot}$ reaction on the solvent [44]. Experiments were also performed with *T. cruzi* microsomes. Whereas primary radicals equivalent to those obtained with liver microsomes and shown in Fig. 2 gave signals of relatively low intensities, products resulting from their reaction with oxygen

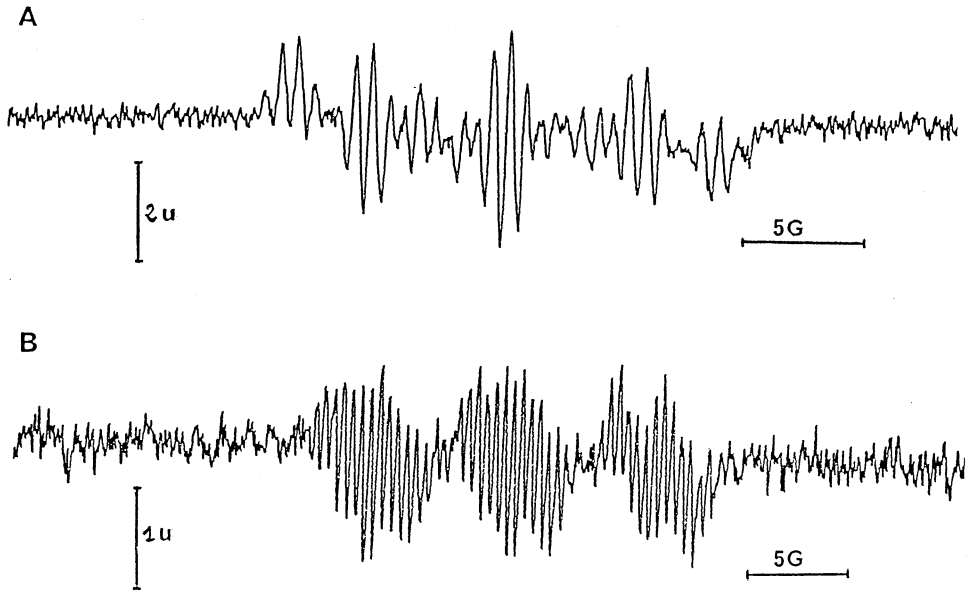

FIG. 2. The spectra of the nitro anion radical from Nifurtimox and Megazol. ESR spectra of the nitro anion radicals observed after an anaerobic incubation at 37° with liver microsomes (4 mg protein/mL), NADPH (1 mM), DTPA (1 mM) in phosphate buffer (20 mM), KCl 150 mM, pH 7.4, and (A) Nifurtimox (1 mM in DMSO 10% v/v) after 4 min of incubation or (B) Megazol (1 mM in DMSO 10% v/v), accumulated during 14 min of incubation. The spectra were obtained with a nominal microwave power of 10 mW and an amplitude modulation of 1.05 G.

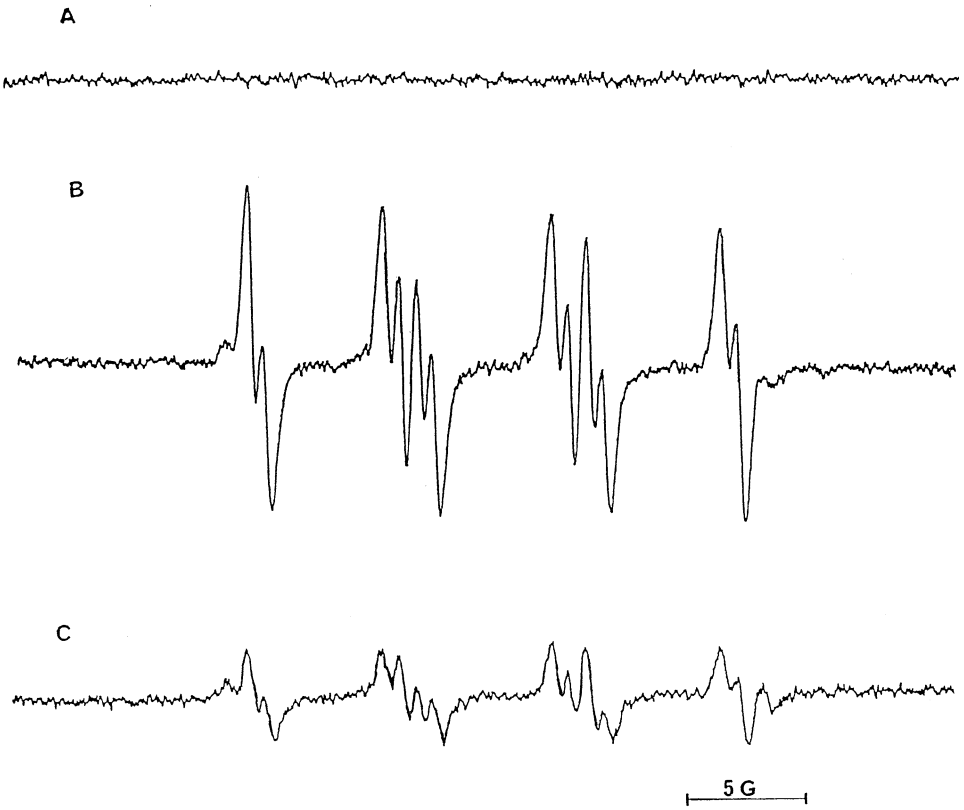

FIG. 3. ESR spectra of the DMPO–$O_2^{\cdot-}$ adducts obtained with NADPH: cytochrome P450 reductase. ESR spectra of the DMPO–$O_2^{\cdot}$ adducts obtained during aerobic incubations of NADPH: cytochrome P450 reductase. The spectra were observed 5 min after incubation at 37° with NADPH: cytochrome P450 reductase (1.44 μg protein/mL), NADPH (1 mM), DTPA (1 mM), DMPO (100 mM) in phosphate buffer (20 mM), KCl 150 mM, pH 7.4, and (A) acetonitrile (10% v/v), (B) Nifurtimox (0.5 mM in acetonitrile 10% v/v), or (C) Megazol (1 mM in acetonitrile 10% v/v). The spectra were obtained with a nominal microwave power of 10 mW and an amplitude modulation of 1.05 G.

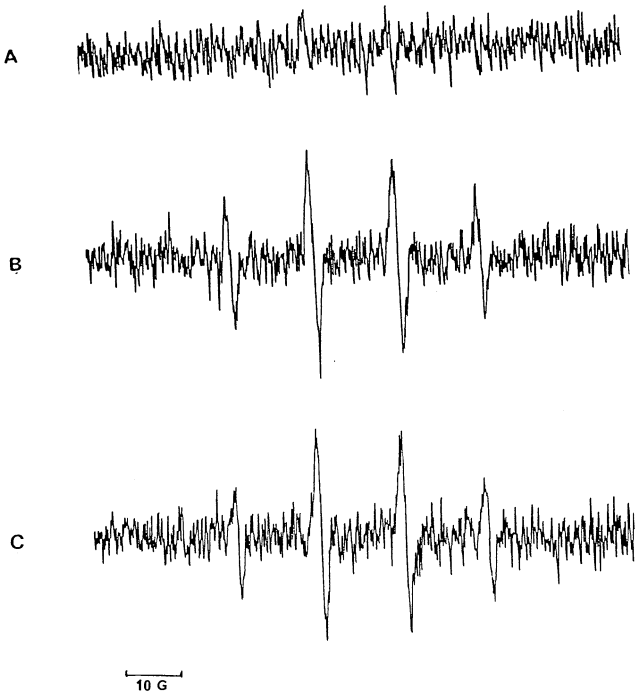

FIG. 4. ESR spectra of the DMPO–OH˙ adducts obtained with *T. cruzi* extracts. ESR spectra of the DMPO–OH˙ adducts obtained during incubations of *T. cruzi* microsomal fraction. The spectra were observed 10 min after incubation at 37° with *T. cruzi* microsomal fraction (4 mg protein/mL), NADPH (1 mM), DTPA (1 mM), DMPO (100 mM) in phosphate buffer (20 mM), KCl 150 mM, pH 7.4, and (A) acetonitrile (10% v/v), (B) Nifurtimox (1 mM in acetonitrile 10% v/v), or (C) Megazol (1 mM in acetonitrile 10% v/v). The spectra were obtained with a nominal microwave power of 10 mW and an amplitude modulation of 1.05 G.

were present. Figure 4 shows the corresponding DMPO–OH˙ adducts generated from Nifurtimox and Megazol, respectively.

Interaction of Megazol and Nifurtimox with GR, LipDH, and TR

Several aromatic nitrocompounds are effective inhibitors and/or redox cycling substrates of GR [45–47], LipDH [14, 22], and TR [33, 48, 49]. In order to gain an insight into the antitrypanosomal mode of Megazol action, the interaction of the nitroimidazole derivate with these flavoenzymes was investigated.

For yeast GR, both Nifurtimox [46] and Megazol acted as uncompetitive inhibitors towards NADPH and glutathione (K_i = 120 μM with Megazol, and 180 μM with Nifurtimox). Neither nitroaromate modified the slope of the Lineweaver–Burk plots for NADPH and glutathione, but they did decrease the k_{cat} values (data not shown, but identical to those presented earlier [45]). The kinetic data may indicate that the compounds do not bind at the NADPH or glutathione binding sites, but in a cavity at the two-fold axis of the homodimeric protein [50]. At this site, riboflavin analogs have been localized [51].

The effect of Nifurtimox on trypanothione reductase is similar to that on GR. The parasite enzyme is inhibited with an IC_{50} value of 200 μM, showing no clear type inhibition [33]. Several nitrofuran derivatives have been found to be potent inhibitors of GR and TR [33, 48, 49], respectively. In contrast, Megazol is not an inhibitor of TR. In the presence of 50 μM TS_2 and 100 μM NADPH, 100 μM Megazol did not influence the enzyme activity. This observation rules out a correlation between the biological activity of Megazol and inhibition of TR, as initially suspected. The finding agrees with previous studies on another nitroimidazole, Benznidazole (Radanil), which also fails to inhibit *T. cruzi* TR.*

The potential inhibition of pig heart and *T. cruzi* LipDH by Megazol was studied both in the forward and the reverse reaction. Under the conditions described, 100 μM Megazol caused less than 5% inhibition.

Another type of interaction between aromatic nitrocompounds and the flavoenzymes is their NAD(P)H-dependent reduction. Under aerobic conditions, the nitrocompound was reduced in a one-electron step to the nitro anion radical which reacted with molecular oxygen, causing the production of superoxide anion radicals (see Fig. 1). Compounds undergoing such a redox cycling have been termed "turncoat inhibitors" [33] or "subversive substrates" [48] because they convert the antioxidative reductase into a pro-oxidative enzyme. The mechanism has been studied in great detail, and *T. cruzi* LipDH has been suggested as being responsible for superoxide production in parasites treated with Nifurtimox [22].

Reduction of Megazol and Nifurtimox by *T. cruzi* LipDH and TR has been studied in comparison to mammalian LipDH and GR, which represent the closest related host enzymes. As summarized in Table I, Megazol was a very weak substrate of the parasite LipDH and TR. The enzymes catalyzed the reduction of the nitroimidazole with second-order rate constants of about 200 M^{-1} sec^{-1}. The reaction led to the generation of superoxide anion radicals as shown by coupling the reaction to cytochrome *c* reduction. With pig heart LipDH, reduction of Megazol was much slower, and in the case of human GR the reaction was negligible. In comparison to Nifurtimox, reduction of Megazol by the flavoenzymes was much slower. The lower rate of Megazol and Benznidazole [14] reduction is probably due to the much slower redox potential of nitroimidazoles in comparison to nitrofurans.

DISCUSSION

The three aromatic nitrocompounds Megazol, Nifurtimox, and Metronidazol are reduced by the different enzymatic systems (Table I). This result is particulary noteworthy in the case of cytochrome b_2, since the reduction potential of cytochrome b_2 is −0.01 V [29]. In other words, reduction is observed even with an unfavorable equilibrium if the

*Krauth-Siegel RL, unpublished results.

reaction is influenced by an irreversible step, in this case the reduction of molecular oxygen. Comparison of k_{cat}/K_m values indicates that Megazol does not undergo a faster enzymatic reduction than the other two compounds, and therefore that its higher biological activity is not related to the first step of the reduction process.

A relationship between the enzyme efficiencies k_{cat}/K_m and the reduction potential of the nitrocompounds was observed in the case of ADR (Table I). The slowest reaction rate was formed with Metronidazole, the compound of lowest redox potential. Obviously, the reduction rates are mainly dependent on electron affinities and not on a structural factor of the different compounds, at least with this enzyme [34].

The single-electron reduction of Megazol was confirmed by ESR studies of the respective radical anions. The generation of the radicals by cytochrome P-450 reductase and rat liver microsomes shows that this reduction proceeds to a significant extent, since the radical anion can accumulate under anaerobic conditions.

Compared to those of Nifurtimox, the nitro anion radical of Megazol and the subsequent DMPO–$O_2^{\cdot -}$ radical appear to have significantly higher half-lives. For the former, this can be understood on the basis of the extended conjugation of the molecule, which is essentially planar [52]; actually this observation more likely corresponds to a slower reduction of Megazol versus Nifurtimox (Table I). As a consequence, the corresponding radical anion is observed over a longer time period. In addition, ESR measurements showed that reductases contained in a *T. cruzi* microsomal fraction are able to generate the radical anion of Megazol characterized by its reaction products with oxygen. There is, therefore, no doubt that this drug is able to interfere with the oxygen metabolism of the parasite, inducing an oxidative stress.

In order to reveal possible target enzymes in the oxygen metabolism, the interaction of Megazol and Nifurtimox with *T. cruzi* LipDH and TR in comparison to mammalian LipDH and GR was studied. Nifurtimox and Megazol are both noncompetitive inhibitors of GR with high K_i values compared to those obtained with effective inhibitors for this enzyme (IC_{50} values in the range of 1 to 20 μM, see ref 53). Inhibition of TR by Nifurtimox (IC_{50} = 200 μM) is even weaker when compared to K_i values in the 0.5–10 μM range for substituted nitrofuran derivates [33, 48, 49] and none with Megazol. The biological activity of Megazol against *T. cruzi* does not rely on the inhibition of this parasite-specific enzyme. *T. cruzi* LipDH as well as TR catalyze the reduction of Megazol and Nifurtimox with the concomitant generation of superoxide anion radicals, whereby Megazol is less efficient than Nifurtimox (Table I). Interestingly, the parasite enzymes catalyze the reduction of Nifurtimox and Megazol much faster than mammalian LipDH and GR (Table I). Since the reaction rates are low, other mechanisms might also contribute to the reduction of the nitrocompounds in parasite [54].

Besides damage induced by hydrogen peroxide accumulation, overproduction of superoxide anion induced by Megazol might also have other detrimental effects for the parasite. It is indeed known that *T. cruzi* has a nitric oxide synthase activity [55] as does the macrophage where it develops. Therefore, peroxynitrite formation resulting from nitric oxide addition on the superoxide anion, a highly damaging species for the parasite [56], should be considered. This effect might be magnified by a concomitant use of superoxide dismutase inhibitors which would increase superoxide anion concentration. Finally, it seems likely that Megazol acts on more than a single target. Recent results [57] indicate that Megazol is an inhibitor of fumarate reductase, an enzyme with an iron–sulfur cluster at the active site. Although the mode of action on this enzyme is unknown, it could involve a possible chelating effect of Megazol on the iron–sulfur cluster. If this is confirmed, a similar effect may be anticipated on other iron–sulfur cluster enzymes such as a ribonucleotide reductase demonstrated in *T. brucei* [58, 59]. In this regard, it is noteworthy that ^{14}C thymidine incorporation by *T. cruzi* cultures is prevented by addition of Megazol in the medium [60].

Although nitroaromatic compounds are probably not the first choice for a drug development because of their often undesirable side effects, Megazol has proved to be suitable for the treatment of Chagas' disease. In addition, the elucidation of the molecular mode of Megazol action will probably reveal new target molecules suited for a rational drug design.

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