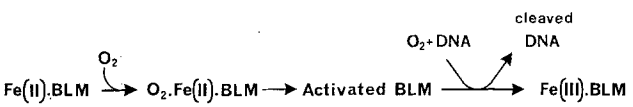

Scheme 2. Mechanism of DNA-cleaving activation of iron-bleomycin (Fe.BLM)[8].

indicating single strand scission, and form III DNA indicating double strand scission. The solutions containing 0.03 µmole of urushiol component (diluted from 0.4 µmole) gave partial cleavage of form I DNA (fig. 2). No differences of activity were seen between the different urushiol components with bleomycin (BLM), activated by various metals, as a mediator[1]. Recent studies suggest that bleomycin and its analogues act by site-selective binding to DNA and oxygen mediated scission of the strands. The mechanism of cleavage is believed to involve activated oxygen, produced as a consequence of oxidation BLM-chelated Fe(II) to Fe(III)[9]. In the case of urushiol, copper catalyzed oxidation of the catechol moiety can lead to the production of activated oxygen which, as described for BLM-Fe, is able to induce DNA breakage (scheme 2). Preliminary experiments show that similar DNA strand scissions can be caused by geranyl- and geranylgeranyl hydroquinone, both dermatotoxic compounds occurring in trichomes of species of *Phacelia* (Hydrophyllacea)[10].

Due to the strong skin irritation and allergic contact dermatitis, elicited by urushiol in vivo, it is somewhat difficult to investigate the actual occurrence of DNA cleavage by urushiol. However, these results suggest that it would be interesting for molecular biologists to design and synthesize a molecule structurally related to urushiol containing a DNA sequence specific recognition pattern[10] linked to a catechol unit through variable length hydrocarbon chain.

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Effects of isoxazolyl-naphthoquinoneimines on growth and oxygen radical production in *Trypanosoma cruzi* and *Crithidia fasciculata*[1]

M. N. Schwarcz de Tarlovsky, S. G. Goijman, M. P. Molina Portela and A. O. M. Stoppani

Bioenergetics Research Centre and Department of Biochemistry, School of Medicine, University of Buenos Aires, Paraguay 2155, 1121-Buenos Aires (Argentina)
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Summary. Several 4-(aminomethylisoxazolyl)-1,2-naphthoquinones inhibited growth and DNA synthesis in *Trypanosoma cruzi* and stimulated O_2 uptake and O_2^- generation by the parasite epimastigotes and their mitochondrial and microsomal membranes; these results support the idea that oxygen radicals play a role in quinone toxicity. Maximal effects on respiration and O_2^- generation were observed with antimycin-inhibited cells. Similar results as well as stimulation of H_2O_2 production were obtained with *Crithidia fasciculata* despite the presence of catalase in this organism.
Key words. Trypanosoma cruzi; *Crithidia fasciculata*; isoxazolyl-naphthoquinoneimines; growth inhibition; DNA synthesis; O_2^- production; H_2O_2 production.

Despite its epidemiological importance, the chemotherapy of Chagas' disease is still an unsolved problem[2]. Many drugs have been assayed for their action on *Trypanosoma cruzi*, the agent of Chagas' disease, but screenings did not include quinoneimines[3,4]. These compounds have similar chemical properties to quinones, including the ability to undergo one electron reduction to give a semiquinone-type free radical and two electron reduction to an aminophenol[5]. Fernandez et al.[6] synthesized a series of isoxazolyl-naphthoquinoneimines (fig. 1), on the assumption that the biological activity of the substituted isoxazol would enhance that of the naphthoquinone group. Therefore, it seemed of interest to establish whether the new compounds affect *T. cruzi* in

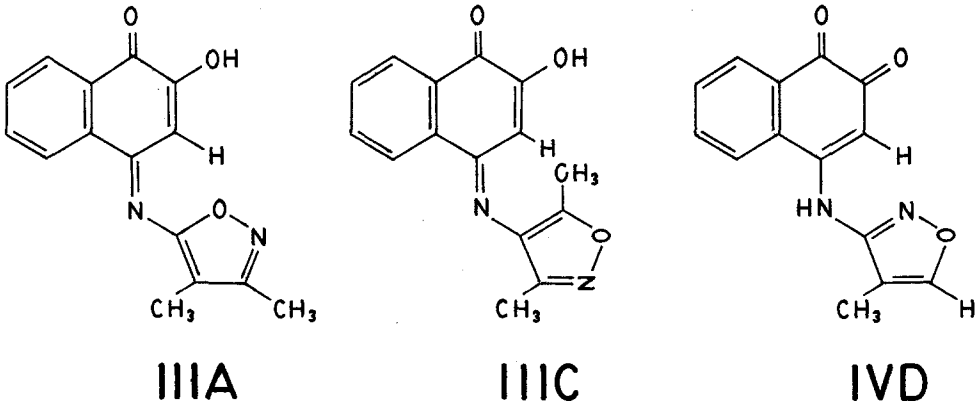

Figure 1. Structure of isoxazolyl-naphthoquinoneimines.

the same way as *o*-naphthoquinones, which are powerful inhibitors of parasite growth and the concomitant synthesis of DNA, RNA and protein[7-12]. The quinoneimines were also assayed on an experimental model allowing pre-screening of potential trypanocidal drugs, namely, *Crithidia fasciculata*[13].

Materials and methods

T. cruzi (Tulahuen strain) was cultured as previously described[7,14,15]. *C. fasciculata* (ATCC 11745) was cultured at 28 °C for 2 days in Bacchi et al.[13] medium. Cell growth was determined by the increased turbidity of cultures, measured with a Klett-Summerson photocolorimeter precalibrated with cell suspensions of known concentration[16]. *T. cruzi* mitochondrial and microsomal membranes were isolated as previously described[7,17,18]. DNA synthesis in *T. cruzi* epimastigotes was measured by the rate of [^{3}H]-thymidine incorporation[14]. The rate of $O_2^{\cdot-}$ generation was measured by the adrenochrome method[7,19]. The incubation mixture contained in a total volume of 3 ml 150 mM KCl, 20 mM KH_2PO_4-K_2HPO_4, pH 7.3, 1.0 mM epinephrine and cells or cell membranes, as indicated under Results. The rate of H_2O_2 generation was measured by the microperoxidase method[20]. The incubation mixture contained 100 mM KCl, 15 mM KH_2PO_4-K_2HPO_4, pH 7.4, 3.5 µM MP-11 microperoxidase and cell protein as indicated under Results. The same methods were used for measuring production of $O_2^{\cdot-}$ and H_2O_2 by quinol oxidation. Oxygen uptake measurements were performed in a Gilson oxygraph, with a Clark or a platinum vibrating electrode, at 30 °C. Other conditions are stated under Results. Catalase was determined spectrophotometrically, at 240 nm, using 10 mM H_2O_2 as substrate[21]. Superoxide dismutase was measured using the xanthine-xanthine oxidase system as $O_2^{\cdot-}$ generator[22]. Enzyme reactions were measured at 30 °C using an Aminco DW 2a UV/VIS or an Aminco Chance spectrophotometer. Other methods were as previously described[7,8,14-16].

Chemicals: Isoxazolyl-naphthoquinoneimines were supplied by Professor M. M. de Bertorello, Department of Pharmacy, School of Chemical Sciences, University of Córdoba, Argentina, and checked for purity by standard methods, including NMR and IR spectroscopy[6]. The reduction of these compounds was performed as described by Boveris et al.[7]. Microperoxidase MP-11, crystallized bovine liver catalase and bovine erythrocytes superoxide dismutase were purchased from the Sigma Chemical Company, St. Louis, MO, USA. Other reagents were as in references[7,8,12,14-16].

Expression of results: Unless stated otherwise, the results presented are the average of duplicate measurements. Experimental values deviated from the mean value by less than 5%.

Abbreviations: The system of Fernandez et al.[6] was used throughout: IIIA, 2-hydroxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine; IIIC, 2-hydroxy-N-(3,5-dimethyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine; IIID, 2-hydroxy-N-(5-methyl-3-isoxazolyl)-1,4-naphthoquinone-4-imine; IIIE, 2-hydroxy-N-(3-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine; IVD, N-(5-methyl-3-isoxazolyl)-1,2-naphthoquinone-4-amine.

Results and discussion

Table 1 shows that isoxazolyl-naphthoquinoneimines inhibited growth and DNA synthesis in *T. cruzi*. These inhibitions were a function of quinone concentration and half-maximal values (growth inhibition experiment) were about 50 µM for IIIA, IIIE and IVD. Quinone redox cycling in epimastigotes was demonstrated by a) the enhancement of oxygen consumption, especially in the presence of antimycin (fig. 2, tracing A); b) the release of $O_2^{\cdot-}$ from epimastigotes to the suspending medium (table 2 and fig. 2C) and c) the effect of superoxide dismutase (fig. 2C). Figure 3 shows the effect of quinone concentration on oxygen uptake by antimycin-inhibited epimastigotes. It can be seen that rate values increased to a

Table 1. Effect of isoxazolyl-naphthoquinoneimines on growth and DNA synthesis in *T. cruzi*

Quinone	Concentration (μM)	Inhibition (% of control value) Cell growth[a]	[^{3}H]-Thymidine incorporation[b]
IIIA	18	36	40
	74	83	73
IIIC	18	12	14
	74	44	37
IIIE	19	21	18
	78	77	--
IVD	19	26	49
	60	79	70

[a] Experimental conditions as described under 'Materials and methods'. [b] Experimental conditions as described in refs 12, 14 and 15; time of incubation, 2 h.

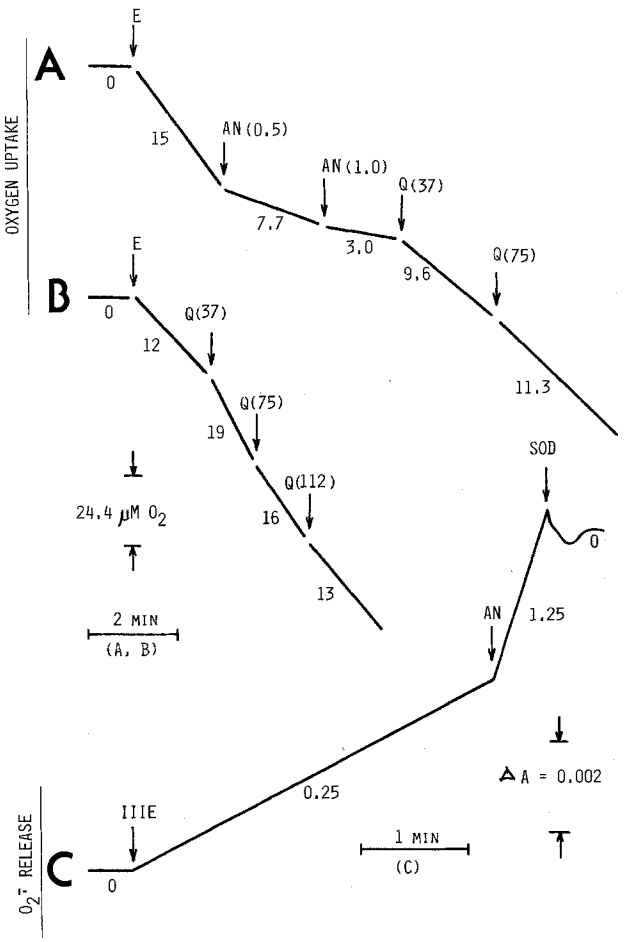

Figure 2. Effect of IIIE on oxygen uptake and O_2^- release by whole *T. cruzi* epimastigotes. *A* and *B*, oxygen uptake measurements. The incubation mixture contained 50 mM KH_2PO_4-K_2HPO_4, pH 7.3, epimastigotes (E, 1.4 mg of protein/ml), antimycin (AN, μg/ml) and IIIE (Q, μM). Antimycin and IIIE were added at the concentrations indicated by the figures in parenthesis. The figures below the tracings indicate oxygen uptake rate (nmol/min/mg of protein). *C*, O_2^- release measurement. The incubation mixture contained epimastigotes (1.2 mg of protein/ml), IIIE (31 μM), antimycin (AN, 0.1 μg/ml) and superoxide dismutase (SOD, 100 U/ml). The figures below the tracing indicate the rate of adrenochrome production (nmol/min/mg of protein). Other conditions were as described under 'Materials and methods'.

maximum and then an inhibitory effect occurred. Moreover, the isoxazolyl group influenced quinone activity, as shown by the different uptake rates induced by IVD, IIIC and IIIE. The effect of antimycin implied quinone reduction by the mitochondrial electron transport, at a site on the substrate side of the antimycin site. This assumption fits in well with the results obtained with mitochondrial membranes (table 2). Under the latter experimental conditions, NADH was a better electron donor than NADPH, as expected from the substrate specificity of mitochondrial NADH-dehydrogenase. Titration of O_2^- production as a function of quinone concentration (6–50 μM IIIC) yielded two K_m values (5.2 and 17 μM, respectively), thus suggesting a complex interaction. *T. cruzi* microsomes contributed also to quinone redox cycling. Thus, with a typical preparation (0.52 mg of protein/ml), 0.42 mM NADPH and 75 μM IIIC (other experimental conditions as in table 2), the rate of O_2^- generation was 1.03 nmol/min/mg of protein, 2.5-fold higher than the value obtained with the mitochondrial membranes (table 2).

Unlike *T. cruzi*[23], *C. fasciculata* contains catalase[24] (table 3 legend). Nevertheless, quinones inhibited growth and stimulated O_2^- and H_2O_2 production by the latter organism, IVD being the most effective on growth (table 3). These results may be explained by the assumption that H_2O_2 generation in *C. fasciculata* occurred in a cell compartment inaccessible to catalase, otherwise the enzyme would have destroyed the H_2O_2, thus preventing either its release to the suspending medium or the production of the highly toxic OH· radical, via the Haber-Weiss and related reactions

$$(O_2^- + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2).$$

The increased rate of O_2^- and/or H_2O_2 production after quinone addition to the assayed organisms involved the

Table 2. Effect of isoxazolyl-naphthoquinoneimines on O_2^- generation by *T. cruzi* epimastigotes and mitochondrial membranes

Experiment	NAD(P)H (0.42 mM)	Quinone	O_2^- generation $\left(\frac{\text{nmol/min}}{\text{mg of protein}}\right)$
A) Epimastigotes	---	IVD	0.28
		IIIA	0.20
B) Mitochondrial membranes	NADH	IVD	1.90
		IIIE	1.24
		IIIA	0.68
C) Mitochondrial membranes	NADH	IIIC	1.10
	NADPH	IIIC	0.34

Samples contained 1.10 (A) or 0.45 (B and C) mg of protein/ml. The mitochondrial membranes were supplemented with NAD(P)H as indicated above; quinone concentration (μM): 50 (A and B) and 32 (C). Other experimental conditions were as described under 'Materials and methods'. In the absence of quinone, respiring *T. cruzi* epimastigotes (Expt. A) did not release O_2^- to the suspending medium. O_2^- generation by mitochondrial membranes (Expts B and C) without added quinone was 0.16–0.34 nmol/min/mg of protein; the corresponding values were subtracted from those in the presence of quinone.

Table 3. Effect of isoxazolyl-naphthoquinoneimines on growth and release of $O_2^{\cdot-}$ and H_2O_2 by *C. fasciculata*

Quinone (10 μM)	Growth inhibition[a] (I_{50}, μM)	Release of[b] $O_2^{\cdot-}$	H_2O_2
IVD	5.5	0	0.29
IIIA	26	1.2	0.16
IIIE	39	1.0	0.08
IIID	42	---	0.10

[a] Experimental conditions were as described under 'Materials and methods'. I_{50}, half-maximal inhibitory concentration. [b] Samples contained 0.11 mg/ml of protein. Other conditions were as described under 'Materials and methods'; values in nmol/min/mg of protein. Superoxide dismutase and catalase in cell homogenates: 1.29 ± 0.60(5) and 0.266 ± 0.05(3) units per mg of protein, respectively (units as described in refs 21 and 22, respectively); mean ± SD; in parenthesis, number of determinations.

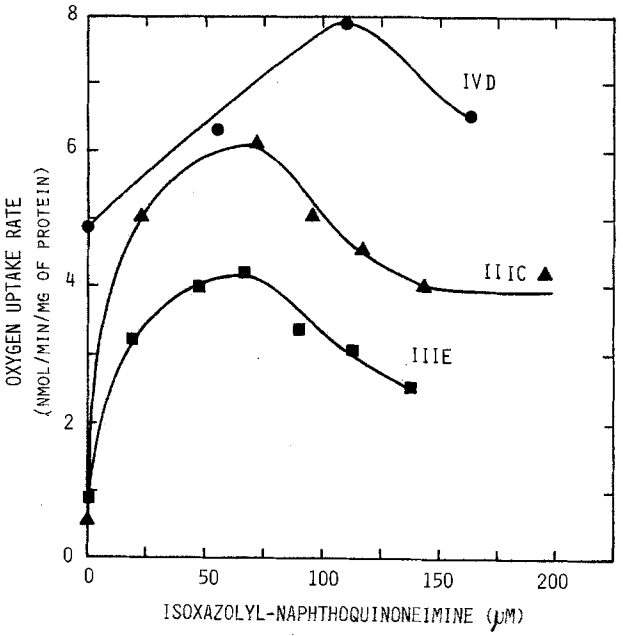

Figure 3. Effect of increasing concentrations of isoxazolyl-naphthoquinoneimines on the oxygen uptake rate by antimycin-inhibited *T. cruzi* epimastigotes. Experimental conditions were as in fig. 2, tracing A (1.0 μg/ml of antimycin).

oxidation of the corresponding quinol (QH_2). Therefore, the rate of quinol autooxidation ($d[O_2^{\cdot-}]/dt = k_3[QH_2][O_2]$; $d[H_2O_2]/dt = k_4[QH_2][O_2]$) was measured as described in Boveris[7]. The results obtained were as follows: a) IVD quinol yielded H_2O_2 but not $O_2^{\cdot-}$ ($k_4 = 3.0\ M^{-1}\cdot s^{-1}$); b) IIIA and IIIE quinols yielded $O_2^{\cdot-}$ but not H_2O_2 ($k_3 = 63$ and $76\ M^{-1}\cdot s^{-1}$, respectively); c) IIIC quinol yielded both H_2O_2 and $O_2^{\cdot-}$ (a single measurement at 20 μM quinol: $O_2^{\cdot-}/H_2O_2$ molar ratio, 2.17). The results with IVD fit in well with those obtained with *C. fasciculata* (table 3) and suggest that in the latter organism the quinone was directly reduced to the quinol, whereas in *T. cruzi* it was reduced to the semiquinone, whose oxidation generated $O_2^{\cdot-}$ (table 2). H_2O_2 production by IIIA and IIIE in *C. fasciculata* (table 3) is easily understood, considering the presence of superoxide dismutase in the latter organism (table 3, legend). The different behaviour of *T. cruzi* and *C. fasciculata* as regards IVD implies metabolic features whose elucidation requires further study. Oxygen radicals might be expected to play a leading role in quinone cytotoxicity; however, the results in figure 3 support direct inhibition of enzyme reactions. In this context, structural differences between molecules (fig. 1 and text) may be relevant, and accordingly it may be assumed that the isoxazolyl moiety modulates quinone reactivity, thus determining the particular effect of each compound in tables 2 and 3, and figure 3. The in vitro effects of isoxazolyl-naphthoquinoneimines on *T. cruzi* epimastigotes, as reported here, resemble those of other lipophilic *o*-naphthoquinones, such as lapachones[7,8,12], although at higher quinone concentrations. Since the usefulness of potential anti-*T. cruzi* drugs depends on their pharmacokinetics and their toxicity for the mammalian host, further studies in vivo are essential for determining the value of isoxazolyl-naphthoquinoneimines for the chemotherapy of Chagas' disease.

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