Menadione kills trophozoites and cysts of *Giardia intestinalis*

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Production of reactive oxygen species by redox cycling in the presence of low levels of oxygen has been studied as a possible approach to anti-protozoal chemotherapeutic strategy. Incubation of the diplomonad flagellate *Giardia intestinalis* with 2-methy-1,4-naphthoquinone (menadione), under anaerobic conditions, gave UV absorption changes characteristic of reduction to menadiol; partial reversal was observed on admitting O2. Under microaerobic conditions, similar to those on the surface of the jejunal mucosa, trophozoites consumed O2 rapidly in the presence of menadione; reaction products included singlet O2 (monitored by single photon counting of O2-dependent low-level chemiluminescence) and H2O2 (measured by the formation of Complex I of microperoxidase). Trophozoites became swollen and incapable of regulatory volume control; these irreversible responses led to loss of motility, cessation of flagellar activity and cell death. Comparison of the sensitivities of trophozoites to metronidazole and menadione gave LC50 values (μg ml−1) of 1·2 and 0·7, respectively; corresponding values for cysts (measured by *in vitro* excystation capacities) were >50 and 1·3. Menadione (LD50 in mice, 0·5 g kg−1) is therefore a potentially more useful and general anti-giardial agent than metronidazole, as it is active against cysts as well as trophozoites.

INTRODUCTION

*Giardia intestinalis* is the most common protozoal intestinal parasite infecting millions of people worldwide, causing serious diarrhoea, growth retardation and malnutrition in children and morbidity in adults (Ortega & Adam, 1997; Lane & Lloyd, 2002). Cysts survive for months in water supplies (Jarroll, 1988) and transmission from infective faeces occurs via contaminated water or by adult-to-adult contact (Rabbani & Islam, 1994). Treatment with anti-giardial agents (nitroimidazoles, nitrofurans or acridine dyes) fails in about 20 % of cases (Ellis *et al*., 1993b) and cysts are highly resistant to water-treatment procedures (Jakubowski & Craun, 2002; Wallis & Campbell, 2002).

The mechanism by which metronidazole, one of the most effective of the nitroimidazoles, kills *G. intestinalis* is not completely understood. Free radical production from nitroimidazoles was demonstrated firstly using liver microsomes (Perez-Reyes *et al*., 1980) and then in the cattle parasite *Trichomonas foetus* (Moreno *et al*., 1983, 1984). In a variety of microaerophilic and anaerobic protists and bacteria, it has been suggested that drug reduction products are responsible for organism killing; these products include the nitro radical anion generated by electron donation from pyruvate : ferredoxin oxidoreductase (PFOR) via ferredoxin species [e.g. in the hydrogenosomes of *trichomonads* (Moreno & Docampo, 1985; Yarlett *et al*., 1986b, 1987)]. Drug free radical generation also leads to the production of reactive oxygen species (ROS) by a redox cycling mechanism. Resistance to metronidazole is known, in some strains of *Trichomonas vaginalis*, to be due to altered levels of PFOR (Kulda *et al*., 1993; Cammack *et al*., 2003), altered redox properties of ferredoxin (Yarlett *et al*., 1986b) or diminished pools of drug radical anions due to elevated intracellular O2 levels (Lloyd & Pedersen, 1985; Yarlett *et al*., 1986a).

Resistant strains of *G. intestinalis* are sometimes also encountered as clinical isolates; one characteristic of certain resistant strains is the presence of elevated NAD(P)H
oxidase activity (Ellis et al., 1993b). An explanation for this observation (Fig. 1) involved the diversion of reducing power away from the site at which drug reduction to the toxic free radical species can occur.

The G. intestinalis trophozoite inhabits the mucosal lining of the upper small intestine, and at this site the O₂ concentration varies from an undetectably low level up to 60 μM (Atkinson, 1980; Paget et al., 1990; Lloyd et al., 2002). Both the intracellular redox balance and fermentative characteristics of the organism are highly dependent on ambient O₂ levels. A further consequence is the opportunity that the intercellular O₂ pool presents for redox cycling of the nitroimidazole drug (Lloyd & Pedersen, 1985). A product of this process is superoxide radical anion species, O₂⁻. It has been suggested that the consequent cascade of ROS may be as, or even more, cytotoxic than the drug nitro radicals themselves. Based on this hypothesis, we have investigated the action of a redox cycling naphthoquinone, 2-methy-1,4-naphthoquinone (menadione), on G. intestinalis and in this report show that, in the presence of microaerobic levels of O₂, it is highly toxic, not only to trophozoites but also to cysts.

**METHODS**

**Organisms.** G. intestinalis Portland-1 strain ATCC 30888 was grown as described previously in a medium containing 0-1 % (w/v) bovine bile and 10 % (v/v) heat-inactivated fetal calf serum (Keister, 1983; Edwards et al., 1989). Organisms were counted on a Fuchs–Rosenthal haemocytometer slide; typically this gave cell numbers of 2 × 10⁶ ml⁻¹ after 2 days growth in stoppered tubes. Viability of trophozoites was assessed by motility and of cysts by their capacity for excystation (Bingham & Meyer, 1979; Paget et al., 1998).

**Harvesting.** After chilling for 20 min in an ice bucket, tubes were inverted several times to dislodge adherent organisms. After counting, cells were harvested at 1000 g (3000 r.p.m.) for 4 min at room temperature in a bench centrifuge (MSE Minor). After washing once with PBS (pH 7-4) and recentrifugation, organisms were finally resuspended in PBS and kept at 4°C.

**Analytical methods.** Absorbance and light scattering measurements (Park et al., 1997) were done in a Beckmann DV 7500 UV/visible spectrophotometer. Oxygen consumption at controlled low O₂ concentrations was in a stirred reaction vessel fitted with a Radiometer (Copenhagen) O₂ electrode in a Hitachi 557 double-beam spectrophotometer (Degn et al., 1980) or used in an EMI single photon counting luminometer fitted with a cooled (~20°C) low-background photomultiplier tube (Lloyd et al., 1979; Biagini et al., 2001). Gas mixtures were made using pre-purified N₂; O₂ and solubility tables were used to calculate dissolved O₂ (Wilhelm et al., 1977). H₂O₂ was measured using microperoxidase (Paget et al., 1987).

**Electron spin resonance (ESR) spectrometry.** Incubation of trophozoites under microaerobic conditions in the cavity of the Varian E109 ESR spectrometer was as described in detail previously (Lloyd & Pedersen, 1985). Organisms, suspended in PBS, were placed in an O₂-permeable silicone rubber catheter tube in the presence of 20 mM glucose and 50 mM metronidazole. The O₂ partial pressure of the mobile gas phase was fixed at a series of values from 0 to 101 kPa, using a digital gas mixing device (Degn et al., 1980).
Materials. Gases were from Air Products. Tryptone was purchased from Becton-Dickinson, and trypicase from BioMérieux. Fetal calf serum was supplied by Gibco-BRL, and Nunclon screw-capped culture tubes were from Life Technologies. All other chemicals were from Sigma-Aldrich-Fluka.

RESULTS

Incubation of a packed suspension of *G. intestinalis* trophozoites with 20 mM glucose and metronidazole in the presence of low levels of O₂ (0–20 kPa) in the cavity of an ESR spectrometer gave no detectable nitro radical anion signal, even though such signals were routinely observed from *T. vaginalis* under identical conditions.

Although metronidazole is one of the most potent anti-giardial agents currently in use (Breccia et al., 1982; Ellis et al., 1993b) its toxicity towards *G. intestinalis* cysts is somewhat limited (Paget et al., 1993, 1998). Therefore, a comparison between the anti-giardial efficacies of the nitroimidazole (metronidazole) and menadione against both developmental stages of the organism was conducted. Against trophozoites, metronidazole and menadione had LC₅₀ values (µg ml⁻¹) of 1·2 ± 0·2 and 0·7 ± 0·2, respectively. Against cysts, the anti-giardial agents had LC₅₀ values of >50 and 1·3 ± 0·3, respectively. Therefore, menadione was much more effective at killing trophozoites and cysts of *G. intestinalis* than metronidazole.

Incubation of 100 µM menadione with *G. intestinalis* trophozoites gave changes in UV absorbance (Fig. 2).

Thus, under anaerobic conditions (sparging with pre-purified N₂), progressive reduction occurred over 25 min (λₘₐₓ shifted from 239 to 226 nm, A₂74 decreased and A₃3₄ increased, spectra 2 and 3). Exposure to O₂ gave partial reversal of these changes (spectrum 4). Organisms initially showed accelerated O₂ consumption when incubated with menadione under microaerobic conditions (Fig. 3a), but

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**Fig. 2.** Changes in absorbance during incubation of *G. intestinalis* trophozoites (4 × 10⁷ organisms ml⁻¹) in PBS pH 6·8 with 10 mM glucose and 100 µM menadione (spectrum 1). After anaerobic incubation under pre-purified N₂ for 25 min (spectra 2, 3), the suspension was oxygenated by bubbling with air for 10 min (spectrum 4). Spectra of menadione and menadiol (complete reduction with NaBH₄) are also shown in the inset.

**Fig. 3.** O₂ consumption and ROS generation by *G. intestinalis* in the presence of menadione. (a) Trophozoites (5 × 10⁶ ml⁻¹) were added to PBS and stirred under a mobile gas phase of 2% O₂ in N₂. Menadione (100 µM) initially gave stimulation of O₂ demand, but by 40 min later complete inhibition of O₂ consumption. (b) H₂O₂ production during menadione-stimulated respiration. Increasing absorbance (419–407 nm) indicates accumulation of microperoxidase/H₂O₂. (c) Chemiluminescence during menadione-stimulated respiration.
prolonged exposure to reaction products eventually gave complete inhibition of O2 demand and loss of motility of the trophozoites. Menadione concentration giving 50% inhibition of O2 uptake was 3 μM. These changes were paralleled by accumulation of H2O2, which could be trapped in the presence of microperoxidase (Fig. 3b) (Paget et al., 1987). Organisms exposed to 10 μM O2 produced H2O2 at a basal rate, and the addition of 10 μM menadione gave a sixfold increase during the phase of accelerated O2 consumption. The O2-dependence of H2O2 generation was also demonstrated in this system. In a similar experiment, we monitored low-level chemiluminescence (Lloyd et al., 1979), characteristic of singlet O2 (Fig. 3c). In the absence of menadione, photon counts from a concentrated suspension of G. intestinalis trophozoites (4 × 10^8 ml^-1) were less than 50 s^-1 at 15 μM O2 and were decreased to <10 s^-1 under an atmosphere of pre-purified N2. Menadione (10 μM) stimulated both O2 consumption and photon emission at 15 μM O2, and switching the mobile gas phase from 0-8 kPa O2 to N2 led to rapid exhaustion of dissolved O2 and decreased chemiluminescence. Re-oxygenation gave an overshoot in light emission before relaxation to the microaerobic steady state.

One consequence of free radical-induced damage is damage to osmoregulation. Fig. 4 shows the effects of menadione on volume regulation in G. intestinalis (Park et al., 1997). Organisms washed and resuspended in PBS 308 m Osm kg^-1 (pH 7-4) maintained their light-scattering characteristics at a stable level over a period of 25 min. Then, dilution by 23% on addition of water gave a phase of swelling (as indicated by decreased light scattering) which was complete after a further 5 min. Cell volume was then subject to a regulatory volume decrease which was completed in a further 20 min. These organisms had maintained their normal size and shape and also showed normal flagellar activity. In the presence of 100 μM menadione, swelling was immediate and, after hypo-osmotic shock, no sustained recovery of volume was indicated and organisms continued to swell for at least 45 min. These menadione-treated organisms had become swollen and distorted, and their flagella were no longer active; many had ruptured. Flow cytometric measurements (forward light scatter) confirmed that organisms became swollen in the presence of menadione; flow cytometry showed that the population behaves homogeneously with respect to decreased forward light scatter (not shown).

**DISCUSSION**

The 5-nitroimidazoles, like the naphthoquinone menadione, have to be reductively activated to radical anion species before their cytotoxic effects are exerted. In another microaerophilic protozoan parasite, T. vaginalis, intracellular generation of the metronidazole radicals has been directly demonstrated by ESR spectrometry (Lloyd & Pedersen, 1985). Identical experiments with G. intestinalis trophozoites failed to reveal an ESR-detectable pool of bioreduced drug; it may be that for metronidazole as with menadione, as shown in this study, cytotoxicity towards this species is mediated via the generation of ROS. Extensive work on the usefulness of redox cycling naphthoquinones as anti-protozoal agents has been reported previously (Croft et al., 1985, 1992; Portela et al., 1996; Portela & Stoppani, 1996; Villamil et al., 1997). Observations reported here suggest that ROS are generated from menadione in Giardia lamblia trophozoites and provide the mechanism whereby menadione leads to rapid cell death. Bioreduction (de Groot et al., 1985) of menadione (Q^2^-), by flavoprotein/iron–sulphur-mediated electron transport in this organism (Ellis et al., 1993a), precedes autoxidation in the presence

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**Fig. 4.** Time-course of absorbance changes in response to hypo-osmotic challenge. G. intestinalis trophozoites suspended in iso-osmotic PBS (308 m Osm kg^-1) at 25 °C in the absence (●) or presence (▲) of 100 μM menadione were diluted (at arrow) to 214 m Osm kg^-1.
of O₂ (Cadenas et al., 1977), and redox cycling, which in turn provides opportunities for generation of ROS.\nu

\[ Q^2− + O₂ → Q^*− + O₂^* \]

\[ Q^*− + O₂ → Q + O₂^* \]

\[ Q^− + O₂ + 2H^+ → Q^*− + H₂O₂ \]

\[ 2Q^*− → Q^2− + Q \]

This in turn provides inter-radical reactions (Halliwell & Gutteridge, 1989; Guillen et al., 1997).

\[ O₂^− + H₂O₂ + H^+ → O₂ + H₂O + OH^* \]

\[ O₂^*− + OH^* → OH− + 1/2O₂ \]

\[ O₂^*− + O₂ → O₂^− + 1/2O₂ \]

\[ O₂^− + 2H^+ → H₂O₂ \]

\[ 2O₂^− → [O₃H₂O₂] → 2O₂ + hv \]

We demonstrate here that both life cycle stages are killed by menadione (LC₅₀ values of 0.7 μM and 1.3 μM for trophozoites and cysts, respectively). The menadione free radical generates H₂O₂, as measured by the formation of Complex I of microperoxidase, and probably also singlet oxygen \(^{1}O₂\), as monitored by single photon counting of chemiluminescence. Trophozoites swell and become incapable of regulatory volume control. Menadione is therefore a potent anti-giardial agent active against cysts as well as trophozoites, and merits further studies as a potentially useful compound for chemotherapeutic treatment of infections.

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