

Anti-*Trichomonas vaginalis* activity of 1,10-phenanthroline-5,6-dione-based metallodrugs and synergistic effect with metronidazole

Research Article

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Abstract

Trichomonas vaginalis is responsible for the most common non-viral, sexually transmitted infection, human trichomoniasis, and is associated with an increased susceptibility to HIV. An escalation in resistance (2.5–10%) to the clinical drug, metronidazole (MTZ), has been detected and this compound also has adverse side-effects. Therefore, new treatment options are urgently required. Herein, we investigate the possible anti-*T. vaginalis* activity of 1,10-phenanthroline-5,6-dione (phendione) and its metal complexes, [Ag(phendione)₂](ClO₄) and [Cu(phendione)₃](ClO₄)₂·4H₂O. Minimum inhibitory concentration (MIC) against *T. vaginalis* ATCC 30236 and three fresh clinical isolates and mammalian cells were performed using serial dilution generating IC₅₀ and CC₅₀ values. Drugs combinations with MTZ were evaluated by chequerboard assay. A strong anti-*T. vaginalis* activity was found for all test compounds. IC₅₀ values obtained for [Cu(phendione)₃](ClO₄)₂·4H₂O were similar or lower than those obtained for MTZ. *In vitro* assays with normal cells showed low cytotoxicity and [Cu(phendione)₃](ClO₄)₂·4H₂O presented a high selectivity index (SI) for fibroblasts (SI = 11.39) and erythrocytes (SI > 57.47). Chequerboard assay demonstrated that the combination of [Cu(phendione)₃](ClO₄)₂·4H₂O with MTZ leads to synergistic interaction, which suggests distinct mechanisms of action of the copper-phendione complex and avoiding the MTZ resistance pathways. Our results highlight the importance of phendione-based drugs as potential molecules of pharmaceutical interest.

Introduction

The emergence of metronidazole (MTZ)-resistant isolates of *Trichomonas vaginalis* (Schwebke *et al.*, 2006), a causative agent of neglected parasitic infections, is considered a real public health problem (Secor *et al.*, 2014). *Trichomonas vaginalis* causes a non-viral, sexually transmitted infection (STI) with an incidence of 276 million new cases each year (WHO, 2012). Several complications are associated with trichomoniasis, such as the acquisition and transmission of HIV (Van Der Pol *et al.*, 2008), pregnancy outcomes, and its relation to prostate and cervical cancers (Menezes *et al.*, 2016). 5-Nitroimidazole drugs, such as MTZ and tinidazole (TNZ), are the only FDA-approved drugs for the treatment of trichomoniasis. MTZ action occurs through enzymatic pathways, and mechanisms of resistance have already been described (Vieira *et al.*, 2017b). The elevated prevalence of infection has been linked to scarce STI control programmes, and the annual cost to the public health bill in the USA alone to treat trichomoniasis and its associated complications has been estimated to be US\$24–167 million (Owusu-Edusei *et al.*, 2013).

Phenanthrenes are the secondary metabolites produced by plants from Orchidaceae. Isolated compounds have been investigated for their anticancer, antimicrobial, anti-inflammatory and antioxidant activities (Tóth *et al.*, 2017). 1,10-Phenanthroline-5,6-dione (phendione), a phenanthrene-based compound, and its associated metal complexes (metal = Cu²⁺, Ag⁺) have been shown to exhibit broad antimicrobial properties (McCann *et al.*, 2004). *In vivo* toxicity studies in *Galleria mellonella* larvae and in mice (acute and chronic toxicity testing) demonstrated that phendione, [Cu(phendione)₃](ClO₄)₂·4H₂O (Cu-phendione) and [Ag(phendione)₂](ClO₄) (Ag-phendione) were well tolerated (McCann *et al.*, 2012). Taking into account the urgency to source new compounds capable of killing trichomonads, the aim of this study was to test the possible anti-*T. vaginalis* activity of phendione, Cu-phendione and Ag-phendione, as well as to evaluate their selectivity and possible synergism when co-administered with MTZ.

Materials and methods

Compounds

phendione, [Ag(phendione)₂](ClO₄) (Ag-phendione) and [Cu(phendione)₃](ClO₄)₂·4H₂O (Cu-phendione) were prepared in accordance with the methods described in the literature (McCann et al., 2004) (chemical structures are given in Supplementary Fig. S1).

Culture of *T. vaginalis*

The assays used *T. vaginalis* ATCC 30236 and three fresh clinical isolates (TV-LACH4, TV-LACM15 and TV-LACM22) obtained from Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia UFRGS, Brazil (UFRGS Research Ethical Committee approved the assays under authorization number 18923). In order to verify if the presence of symbiosis with *Mycoplasma hominis* (MH) and *Trichomonasvirus* species (TVV) could be related to the test compounds' susceptibility, isolates were selected according to the following: harbouring TVV only (TV-LACH4), MH only (TV-LACM22), both organisms (ATCC 30236), or not harbouring MH neither TVV (TV-LACM15). Trophozoites were maintained in TYM medium supplemented with heat-inactivated adult bovine serum (10%, v/v) at 37 °C (Diamond et al., 1957). Trichomonads in the logarithmic growth phase with normal morphology were used in the assays.

Minimum inhibitory concentration (MIC) and IC₅₀ determination

Test compounds, MTZ, and the simple metal salts, AgNO₃ and CuSO₄·5H₂O, at decreasing concentrations from 100 μM (mg L⁻¹ concentration in Table 1), and 2 × 10⁵ trophozoites mL⁻¹ were incubated at 37 °C, 5% CO₂ atmosphere for 24 h. After incubation, viability was determined by counting trophozoites with a haemocytometer using trypan blue exclusion dye (0.2%). MIC was determined by inoculation of trophozoites in fresh medium without compounds and analysed for 5 days to confirm the absence of parasite growth (Vieira et al., 2017a).

Effect of compounds on *T. vaginalis* growth kinetic

Trichomonads (ATCC 30236) at a density of 2 × 10⁵ trophozoites mL⁻¹ were incubated in TYM supplemented with the compounds at MIC and IC₅₀ values. Trophozoites were counted with a haemocytometer at different time periods (from 2 to 120 h).

Cytotoxicity and CC₅₀ determination

HMVII (a tumour lineage from human vaginal epithelial cells) and 3T3-C1 (a non-tumour murine fibroblast lineage) were used. The assay dilution was performed as described above using RPMI and DMEM media, respectively, supplemented with FBS (20%, v/v). After 24 h of exposure, viability was assessed through [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]bromide (MTT) assay and the formazan produced by viable cell was spectrophotometrically measured at 570 nm (Hübner et al., 2016). MIC was determined comparatively with a negative control (Triton X-100). The selectivity index (SI) for each mammalian cell was calculated based on the ratio CC₅₀/IC₅₀, using the IC₅₀ value calculated by geometric mean among different *T. vaginalis* isolates.

Haemolytic assay

To evaluate the toxic effect of the test compounds and MTZ on human erythrocytes, haemolysis experiments were performed. The UFRGS Research Ethical Committee approved the assays

Table 1. Anti-*Trichomonas vaginalis* activity and cytotoxicity effect of MTZ, phendione and its metal complexes

	Trichomonas vaginalis isolates										Mammalian cells							
	ATCC 30236		TV LAC-M15		TV LAC-M22		TV LAC-H4		Geometric mean (μM)		HMVII		3T3-C1		Haemolysis			
	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	CC ₅₀	SI	CC ₅₀	SI	
Phendione	21.02 (100)	3.57 ± 0.51 (17.0)	2.63 (12.50)	0.68 ± 0.04 (3.25)	10.51 (50)	0.98 ± 0.07 (4.68)	10.51 (50)	1.52 ± 0.11 (7.22)	42.04	6.57	>10.51 (>50)	1.64 ± 0.27 (7.80)	1.19	>10.51 (>50)	9.34 ± 0.97 (44.43)	6.76	>10.51 (>50)	>7.61
Ag-Phendione	31.38 (50)	2.81 ± 0.09 (4.48)	7.85 (12.50)	0.79 ± 0.06 (1.26)	7.85 (12.50)	0.95 ± 0.17 (1.52)	15.69 (25)	4.73 ± 0.31 (7.53)	21.02	2.84	>31.38 (>50)	3.67 ± 0.69 (5.84)	2.06	>31.38 (>50)	12.89 ± 2.15 (20.53)	7.23	>31.38 (>50)	>17.61
Cu-Phendione	12.06 (12.50)	0.81 ± 0.02 (0.84)	6.03 (6.25)	0.48 ± 0.09 (0.50)	6.03 (6.25)	0.42 ± 0.09 (0.44)	12.06 (12.50)	2.00 ± 0.07 (2.07)	8.84	0.87	>48.25 (>50)	3.02 ± 0.77 (3.13)	3.60	>48.25 (>50)	9.56 ± 0.31 (9.91)	1.139	>48.25 (>50)	>57.47
MTZ	0.19 (1.14)	0.07 ± 0.01 (0.40)	4.28 (25)	0.18 ± 0.01 (1.06)	2.14 (12.50)	1.10 ± 0.07 (6.43)	4.28 (25)	0.46 ± 0.07 (2.67)	9.71	1.64	>8.56 (>50)	>8.56 (>50)	>30.49	>8.56 (>50)	>8.56 (>50)	>30.49	>8.56 (>50)	>30.49

Data are the mean ± s.d. of at least three different experiments performed in triplicate. All results are expressed in mg L⁻¹ or in μM (parenthesis), except for SI values.

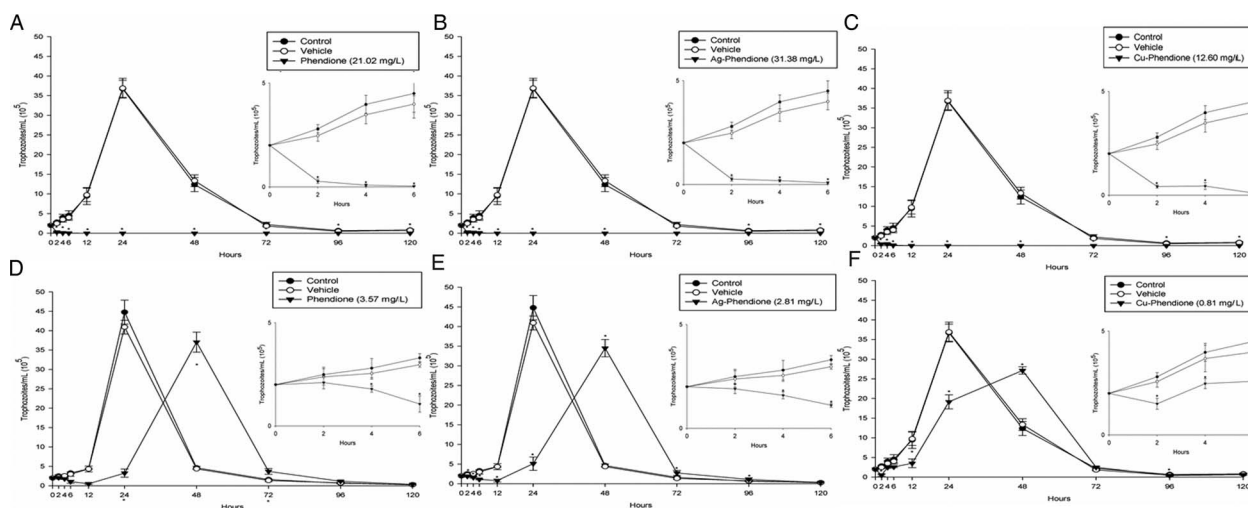


Fig. 1. Growth kinetic curves of ATCC 30236 *T. vaginalis* isolate in the presence of: phendione MIC (A) and IC₅₀ (D); Ag-phendione MIC (B) and IC₅₀ (E); and Cu-phendione MIC (C) and IC₅₀ (F). Treated trophozoites were compared with control (untreated). Insets show data at 0 to 6 hours in detail. Data are the mean \pm s.d. of at least three different experiments (parasite suspensions) performed in triplicate. *Means statistical difference from controls ($P < 0.05$).

under authorization CAAE 69979817.5.0000.5347. Erythrocyte suspension (5×10^7 cells mL⁻¹) was incubated with decreasing concentrations of compounds, starting at 50 μ M (mg L⁻¹ concentration in Table 1), for 24 h at 37 °C (Kiss *et al.*, 2010). Haemoglobin released into the supernatants was quantified spectrophotometrically at 540 nm. Percentage of haemolysis was compared with 100% for the positive control (Triton X-100, 0.2%). SI was also calculated as described earlier.

Chequerboard assay

Trichomonas vaginalis TV-LACM15 isolate was used to check MTZ and Cu-phendione interaction at concentrations: $\frac{1}{4} \times$ IC₅₀, $\frac{1}{2} \times$ IC₅₀, IC₅₀, $2 \times$ IC₅₀ and $4 \times$ IC₅₀. Fractional inhibitory concentration (FICI) was estimated using the following formula: $FIC_A + FIC_B = FICI$, where FIC_A is the value of Cu-phendione in the combination/value of Cu-phendione alone and FIC_B is the value of MTZ in the combination/value of MTZ alone. The interaction was classified as 'synergy' if $FICI \leq 0.5$, 'antagonism' if $FICI > 4.0$ and 'no interaction' if $FICI = 0.5-4.0$ (Odds *et al.*, 2003).

Statistics

All experiments were performed in triplicate with three independent cultures ($n = 3$). Statistical analysis used Student's *t*-test with the 5% level of significance being applied to data. IC₅₀ and CC₅₀ values were calculated using the GraphPad Prism6 software (San Diego, CA) by nonlinear regression.

Results

Anti-*T. vaginalis* activity

Table 1 summarizes the MIC and IC₅₀ values obtained after the treatment (24 h) of different *T. vaginalis* isolates, which present distinct phenotypic backgrounds, with MTZ, phendione, Ag-phendione and Cu-phendione. Overall, all of the test compounds had a strong effect on trophozoite viability, displaying low MIC and IC₅₀ values, and with Cu-phendione having the highest anti-*T. vaginalis* activity. Corroborating these results, the anti-*T. vaginalis* activity of the compounds (at their MIC and IC₅₀ values) was evidenced from kinetic curves (Fig. 1), and 4 h was sufficient to observe significant differences in proliferation

rates between untreated and treated trophozoites. The simple metal salts, AgNO₃ and CuSO₄·5H₂O, were ineffective against the trophozoites (data not shown).

Cytotoxicity assays

Cytotoxicity against HMVII and 3T3-C1 cell lines is shown in Table 1. Cu-phendione showed the highest SI for both erythrocytes (>57.47) and the non-tumour cell lineage (11.39), demonstrating both selectivity towards the parasite and the safety of the complex.

Synergy potential of Cu-phendione with MTZ

Considering the higher MTZ resistance presented by TV-LACM15 (MIC = 4.28 mg L⁻¹) when compared with the ATCC 30236 isolate (MIC = 0.18 mg L⁻¹), TV-LACM15 was used to test the Cu-phendione and MTZ association. The chequerboard assay revealed a synergistic effect ($FICI \leq 0.5$) upon co-administration of Cu-phendione and MTZ (Table 2).

Discussion

The treatment of trichomoniasis relies on a single class of drugs, 5-nitroimidazoles, which present several adverse effects and are failing due to emerging resistance. Studies demonstrated an inefficient effect of MTZ, result of adverse reactions that decrease or impair treatment adherence by the patient. Cases of hypersensitivity reaction were already described leading to severe anaphylactic reactions and disulfiram-like alcohol intolerance, beyond the commonly effect of headache, nausea, vertigo, vomiting, diarrhoea and a metallic taste (Kissinger, 2015). Moreover, progress of resistance to the 5-nitroimidazole class is spread worldwide and is of concern for the public health. A recent study in the USA demonstrated that association between trichomoniasis and HIV infection is a burden to the health system, with costs reaching \$167 million per year (Chesson, 2004).

Scientific development in new alternatives for the treatment of trichomoniasis is based on natural and synthetic compounds in order to discover new promising activity. Vieira *et al.* (2015) gathered different sources of molecules currently studied, including those derived from marine products and other from native flora, such as plants of the Brazilian Caatinga bioma and used by

Table 2. FICI data for *T. vaginalis* TV-LACM15 isolate for each combination tested

Cu-phendione concentration (mg L ⁻¹)	MTZ concentration (mg L ⁻¹)				
	0.05	0.09	0.18	0.36	0.72
0.12	0.74	1.42	1.36	1.38	1.89
0.24	1.54	1.47	1.06	1.22	3.04
0.48	1.19	1.22	1.01	1.19	1.05
0.97	0.75	0.82	0.91	0.51	0.87
1.93	0.93	0.70	0.38 ^a	0.17 ^a	0.04 ^a

^aSynergy interaction by Odds (2003).

indigenous tribes. These natural compounds or semisynthetic derivatives generate about 35% of the new approved drugs (Newman, 2012). Concerning about synthetic products against *T. vaginalis*, Bala & Chhonker, (2018) brought together several trichomonocidal derivatives of 5-nitroimidazoles, benzimidazole, amine, isatin, in addition to agents already approved and microbicide with spermicidal or antifungal properties.

Metal-based drugs are already used in therapies as cisplatin with platinum for cancers' treatment and gold-based drugs as auranofin in rheumatoid arthritis (Allardycy & Dyson, 2016). Indeed, auranofin has been demonstrated anti-*T. vaginalis* effectiveness *in vitro* and *in vivo*, presenting IC₅₀ values of 0.4–2.5 μM (Hopper et al., 2016). In this context, we highlight the metal-based compound Cu-phendione, which demonstrated high activity against this parasite, with MIC value of 8.84 μM and IC₅₀ 0.87 μM, and more effective than MTZ.

Phendione and its metal complexes have previously demonstrated antimicrobial properties against *Candida albicans* yeast (Eshwika et al., 2004), the multi-resistant mould *Scedosporium apiospermum* (McCann et al., 2012), dematiaceous *Phialophora verrucosa* (Granato et al., 2017) and the Gram-negative bacterium *Pseudomonas aeruginosa* (Viganor et al., 2016). Herein, we have demonstrated the potent activity of this class of compound against *T. vaginalis*. Table 1 shows IC₅₀ values very close to or lower than that of the clinical drug, MTZ. Each *T. vaginalis* isolate used has characteristics previously evaluated by Becker et al. (2015), such as the occurrence of symbiosis infections with *MH* and *TVV*. As expected, no relation between anti-*T. vaginalis* activity and symbiosis was found, confirming the antiparasitic activity against several isolate types. Growth kinetics demonstrated that an exposure time of only 4 h was necessary to reduce the number of viable trophozoites, and at 12 h there was a complete cessation of parasite proliferation at the MIC values. The greater activity of the metal–phendione complexes, compared with the simple metal salts (AgNO₃ and CuSO₄·5H₂O) and the metal-free phendione ligand, against *T. vaginalis* trophozoites highlights the necessity for elucidating their mechanisms of action in further studies.

The pathogen *T. vaginalis* initiates the infection by recognition of host cells, a process mediated by cysteine proteases located on the parasite surface. Once in the site of infection, trophozoites alter their conformation from pyriform to amoeboid for cytoadherence. This tight association supports the process of tissue damage to ensure their survival by acquisition of nutrients, as iron and lipids, from erythrocytes (Menezes et al., 2016). Thus, investigating the cytotoxicity of bioactive compounds against cells involved in the infection is crucial. The present compounds presented a low SI against HMVII vaginal epithelial cells, as expected, since earlier studies showed them to have exceptional *in vitro* anti-cancer activity (McCann et al., 2012). In tests using the erythrocytes and the non-tumour cell line 3T3-C1, Cu-phendione

was well tolerated and comparable with the reference drug, MTZ, which reveals pharmacological selectivity. Furthermore, phendione and its metal complexes caused no haemolysis. These preliminary results suggest that the compounds, particularly Cu-phendione, were well tolerated *in vitro* by host cells.

Cu-phendione, the compound with the highest anti-*T. vaginalis* activity and the best SI, was selected to check for synergistic interactions with MTZ using the checkerboard assay. A synergy effect was observed using Cu-phendione (≥1.93 mg L⁻¹) in combination with MTZ (≥0.18 mg L⁻¹), within a range of drug concentrations that are well tolerated by mammalian cells (Table 1). These results demonstrate that the interaction between both compounds reduces the concentration required to cause parasite death and suggest different sites of action for the two compounds. Consequently, distinct mechanisms may be involved in cell death thus enabling evasion of the MTZ resistance pathways.

There is an urgent necessity for new therapeutics capable of effectively treating trichomoniasis and severing the link between *T. vaginalis* infection and HIV transmission. In this context, Cu-phendione offers credible potential against the proliferation of *T. vaginalis*. Future studies will focus on the elucidation of the mechanism(s) of action of these compounds.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S003118201800152X>.

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Conflicts of interest. None.

Ethical standards. The *T. vaginalis* fresh clinical isolates were obtained from Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia UFRGS, Brazil (UFRGS Research Ethical Committee approved the assays under authorization number 18923) and the human erythrocytes for the haemolysis experiments were obtained from healthy volunteers (UFRGS Research Ethical Committee approved the assays under authorization CAAE 69979817.5.0000.5347).

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