Hydrogenosome Metabolism Is the Key Target for Antiparasitic Activity of Resveratrol against *Trichomonas vaginalis*

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Metronidazole (MDZ) and related 5-nitroimidazoles are the recommended drugs for treatment of trichomoniasis, a sexually transmitted disease caused by the protozoan parasite *Trichomonas vaginalis*. However, novel treatment options are needed, as recent reports have claimed resistance to these drugs in *T. vaginalis* isolates. In this study, we analyzed for the first time the *in vitro* effects of the natural polyphenol resveratrol (RESV) on *T. vaginalis*. At concentrations of between 25 and 100 μ M, RESV inhibited the *in vitro* growth of *T. vaginalis* trophozoites; doses of 25 μ M exerted a cytostatic effect, and higher doses exerted a cytotoxic effect. At these concentrations, RESV caused inhibition of the specific activity of a 120-kDa [Fe]-hydrogenase (Tvhyd). RESV did not affect Tvhyd gene expression and upregulated pyruvate-ferredoxin oxidoreductase (a hydrogenosomal enzyme) gene expression only at a high dose (100 μ M). At doses of 50 to 100 μ M, RESV also caused overexpression of heat shock protein 70 (Hsp70), a protective protein found in the hydrogenosome of *T. vaginalis*. The results demonstrate the potential of RESV as an antiparasitic treatment for trichomoniasis and suggest that the mechanism of action involves induction of hydrogenosomal dysfunction. In view of the results, we propose hydrogenosomal metabolism as a key target in the design of novel antiparasitic drugs.

Trichomoniasis is known as the most common nonviral sexually transmitted disease (STD) in the world (1). An ancient extracellular obligate parabasal flagellate, *Trichomonas vaginalis*, which colonizes the human urogenital tract, causes the disease. Trichomoniasis is a frequent source of vaginitis (about 4 to 35% of cases of vaginitis diagnosed in symptomatic women) and may evolve from an asymptomatic to a chronic inflammatory disease (2, 3, 4). The disease can contribute to premature rupture of membranes during pregnancy, preterm birth, and low birth weight and may facilitate HIV acquisition (5). Currently, metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole), which are potent drugs against infections caused by anaerobic or microaerophilic microorganisms, are the only recommended drugs for standard treatment of *T. vaginalis* infection. However, resistance of *T. vaginalis* to MDZ, allergic reactions, and failure to remedy the infection with two consecutive courses of treatment have been reported (4, 6–9). Studies have shown that at least 5% of clinical cases of trichomoniasis are caused by parasites that are resistant to the above-mentioned drugs. Because of the lack of approved alternative treatments, the only option for patients with resistant infections is to use higher and sometimes toxic doses of MDZ, which leads to an increase in the occurrence of side effects (6). The reliance on a single class of drugs for treatment of *T. vaginalis* infections may be problematic if resistance to nitroimidazole becomes widespread in *T. vaginalis* strains. Hence, studies to find new, safe agents that are efficacious in the treatment and prevention of refractory trichomoniasis are clearly essential.

Natural products research provides a wide variety of lead structures that are used by the pharmaceutical industry as templates in the development of new drugs that are more effective and have fewer or no undesirable side effects compared to current treatments (10–12). Resveratrol (RESV) is a natural major phytoalexin (a functionally defined class of secondary metabolites), which is produced *de novo* by plants in response to stress factors, such as pathogen attack, and which promotes disease resistance (13). RESV, which is found naturally in grapes and red wine (14), acts as

a precursor for stilbene compounds of higher fungotoxicity that accumulate in grapevine as a result of infection or stress (15). RESV has also been shown to be active against bacteria, fungi, protozoa, and viruses (16–22). In addition to its antimicrobial activity, RESV has also attracted attention on the basis of its health benefits to humans, which include anti-inflammatory effects; reduced risks of cardiovascular disease, cancer, obesity, diabetes, and neurodegenerative diseases; and also extension of life span by mimicking the caloric restriction effect (23).

T. vaginalis is an amitochondrial anaerobic parasite that possesses a hydrogenosome (a double-membrane-bound organelle involved in catabolic processes, including glycolysis, which produce energy and excrete molecular hydrogen, thus aiding redox balance) (24). The pathway of oxidative decarboxylation of pyruvate in hydrogenosomes is responsible for metabolic activation of 5-nitroimidazole drugs, such as MDZ, used to treat trichomoniasis (25). Hydrogenosomes are considered an excellent drug target because their metabolic pathway is distinct from those found in mitochondria, and thus, medicines directed at these organelles will probably not affect the host cells (26).

In this study, we evaluated, for the first time, the *in vitro* effects of RESV on *T. vaginalis*, and we describe some of the potential biochemical targets of hydrogenosomal metabolism involved in the antiparasitic activity.

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MATERIALS AND METHODS

Parasites. The Tv1 isolate of *T. vaginalis* was obtained from a female patient suffering from vaginal trichomoniasis attending the gynecology service at the Santiago de Compostela University Hospital Complex (Spain). Parasites were cultured axenically *in vitro* in modified Diamond's medium (MDM) (27). The parasites were cultured at 35°C in 15-ml culture plates completely filled with the medium, which contained 2% (wt/vol) Trypticase, 1% (wt/vol) yeast extract, 0.5 (wt/vol) maltose, 0.1% (wt/vol) L-ascorbic acid, 0.1% (wt/vol) L-cysteine, 0.1% (wt/vol) KCl, 0.1% (wt/vol) KHCO₃, 0.1% (wt/vol) KH₂PO₄, 0.1% (wt/vol) K₂PO₄, and 0.02% (wt/vol) FeSO₄ (pH 6.2) and was supplemented with 10% (vol/vol) heat-inactivated bovine serum. Cells were grown to late log phase (1 × 10⁶ to 2 × 10⁶ cells/ml) and harvested by centrifugation (200 × g) for all further manipulations.

***In vitro* growth assays.** The effects of RESV and MDZ on the *in vitro* growth of *T. vaginalis* were determined as previously described, with minor modifications (18). For all experiments, stock solutions of RESV and MDZ were prepared in dimethyl sulfoxide (DMSO), to a concentration of 100 mM, and stored away from light at −80°C. In order to investigate their effects on *T. vaginalis*, RESV and MDZ were added to wells of sterile 24-well culture plates (Corning) containing 7 × 10⁴ trophozoites/well in 1 ml of MDM with different concentrations of the drugs (25, 50, and 100 μM for RESV and 6.25, 12.5, 25, 50, and 100 μM for MDZ). Control wells containing DMSO at the highest concentration used were included in each plate. The plates were then incubated for 2 days at 35°C in a container under a vacuum. The number of ciliates was determined daily by counting the numbers in 10-μl aliquots of the medium removed from each well, in a hemocytometer.

Preparation of cell crude extracts (CEs). *T. vaginalis* trophozoites (10⁷) were harvested by centrifugation (200 × g for 5 min at 4°C) and washed twice with Dulbecco's phosphate-buffered saline (DPBS) (pH 7.0) supplemented with calcium and magnesium, discarding the supernatant. Cells were lysed by adding 200 μl of ultrapure water containing 25 mM metrizamide (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (PMSF) to the pellet for 15 min at 4°C. Proteins were quantified by using the Bio-Rad DC assay.

Spectrophotometric assays for [Fe]-hydrogenase activity. *T. vaginalis* [Fe]-hydrogenase (Tvhyd) activity in CEs was quantified spectrophotometrically. Reduced methyl viologen (MV) and a proton (buffer solution) were used as the electron donor and acceptor, respectively, to measure the proton reduction activity (2H⁺ + 2e[−] → H₂). The reaction buffer contained 20 mM sodium dithionite and 1 mM MV dissolved in DPBS. N₂ gas was bubbled through the reaction solution for 10 min in tubes with overflange stoppers. Reaction solution (1.4 ml) was preheated to 37°C and transferred into a cuvette, which was then covered with a silicone plug. The reaction was started by the injection of 0.1 ml of CE with an insulin syringe, and it was monitored at 604 nm in a spectrophotometer (Jenway, United Kingdom) equipped with temperature control. One enzymatic unit of Tvhyd was defined as the amount of enzyme required to reduce 1 μmol of MV (equivalent to the production of 1 μmol of H₂ per min from reduced MV). The specific activity was defined as the number of enzymatic units per milligram of protein.

Tvhyd activity determined by native polyacrylamide gel electrophoresis (PAGE). Aliquots (475 μg) of CE proteins were dissolved in 200 μl of native protein loading sample buffer (0.1 M Tris-HCl [pH 6.8], 20% glycerol, 0.2% bromophenol blue), and 30 μl of sample was loaded onto a 12.5% nondenaturing polyacrylamide gel in a buffer containing 25 mM Tris-HCl, 192 mM glycine (pH 8.3), and 25 mM metrizamide. After electrophoresis, the lane for activity staining was cut out and soaked in basal buffer containing 5 mM MV and 5 mM sodium dithionite previously bubbled for 10 min with N₂ gas. The N₂ gas was then removed and replaced with hydrogen by bubbling H₂ gas through the buffer containing the gel until the Tvhyd band activities were revealed. The staining was fixed by adding 1 mg/ml of 2,3,5-triphenyltetrazolium chloride to the buffer and incubating the gel for 15 min at room temperature; the gel was

then thoroughly washed with deionized water. The bands exhibiting hydrogenase activity stained red due to the formation of a red precipitate.

Functional hydrogenosome alterations. In order to determine if RESV and MDZ cause alterations in hydrogenosome functionality, 2 × 10⁶ *T. vaginalis* trophozoites were incubated with MDM alone or with RESV (50 and 100 mM) or MDZ (6.25 and 12.5 mM) for 2 h at 37°C. The trophozoites were permeabilized with 0.1 mM digitonin and incubated for 2 min at 37°C. The cells were then washed twice by centrifugation (200 × g for 5 min) before being resuspended in 3 ml of MDM containing 500 nM MitoTracker Deep Red FM (Molecular Probes) and incubated for 30 min at 37°C in the dark. After two washes with DPBS by centrifugation, the pellet was resuspended in 100 μl of DPBS, and the fluorescence was measured in a microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA) (excitation/emission = 644/665 nm). In all experiments, the fluorescence produced by spontaneous oxidation of the probe was subtracted from the fluorescence values obtained for the experimental samples. The trophozoites were fixed with 2 to 4% formaldehyde for 15 min and washed twice with DPBS to permeabilize them prior to visualization by fluorescence microscopy. Samples fixed in this way can be used for immediate viewing with a fluorescence microscope (excitation/emission = 644/665 nm) or stored in the dark for several days.

Hydrogenosomal membrane potential. The hydrogenosomal membrane potential (Δψ_m) of *T. vaginalis* was determined by using the JC-1 kit (Molecular Probes) with the cationic fluorescent probe 5,5',6,6'-tetrachloro-1, 1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). JC-1 can selectively enter hydrogenosomes and, according to the magnitude of the hydrogenosomal membrane potential, change its oligomeric state, thereby allowing it to fluoresce (28). The assay was carried out as follows: *T. vaginalis* trophozoites were suspended in culture medium (5 × 10⁵ trophozoites per 100 μl) containing RESV (at concentrations of 0, 25, 50, and 100 mM) or MDZ (at concentrations of 0, 3.125, 6.25, and 12.5 mM) and incubated in 96-well cell culture plates for 2 h at 37°C in a container to which a vacuum was applied. After this period, 10 μl of the probe (diluted 1:10 in MDM) was added to each well, and trophozoites were incubated at 37°C in darkness for 30 min. RESV and MDM were then removed by centrifuging the plates (200 × g for 5 min), and the trophozoites were washed twice with 200 μl of the previously prepared assay buffer and suspended in 100 μl of the same buffer. Finally, emitted fluorescence was measured immediately in a microplate fluorometer to detect fluorescein isothiocyanate (FITC) (excitation/emission = 485/535 nm). In all experiments, the fluorescence produced by spontaneous oxidation of the probe was subtracted from the fluorescence values obtained for the experimental samples.

Assay of intracellular ROS production. Intracellular production of reactive oxygen species (ROS) was evaluated with the fluorescent probe 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA; Fluka). A stock solution of probe (10 mM) was prepared in DMSO (Sigma) and stored in the dark at −80°C until use. The flagellates obtained from cultures were centrifuged at 200 × g for 5 min and then resuspended in MDM (5 × 10⁶ cells/ml). For the assay, 100 μl of the flagellate solution was added to each well of 96-well flat-bottom microtiter plates along with 1 μl of a 1:10 dilution (in DPBS) of stock solution of the probe in DMSO (final concentration of DMSO, 10 μM). To determine the effect of RESV and MDZ on intracellular ROS production, 1 μl of different dilutions of the drugs being tested was also added to provide the final concentrations used (0, 25, 50, and 100 μM for RESV and 0, 3.125, 6.25, and 12.5 μM for MDZ). The plates were then incubated for 1 h at 37°C. Fluorescence was measured in a microplate fluorescence reader (excitation/emission = 490/525 nm). In all experiments, the fluorescence produced by spontaneous oxidation of the probe was subtracted from the fluorescence values obtained for the experimental samples.

RT-qPCR. Total RNA from *T. vaginalis* trophozoites (10⁷ cells/sample) was isolated with a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's instructions. The resulting RNA was dried and dissolved in diethylpyrocarbonate (DEPC)-

FIG 1 Effect of RESV at concentrations of 25, 50, and 100 μ M (A) and MDZ at concentrations of 6.25, 12.5, 25, 50, and 100 μ M (B) on the *in vitro* kinetics of growth of *T. vaginalis*. Each point on the lines represents the mean $\pm$ standard error of the number of trophozoites/ml ($n = 5$). Asterisks indicate statistically significant differences between treated groups and untreated controls (*, $P < 0.01$).

treated RNase-free water at a concentration of 1 μ g/ml. cDNA synthesis (25 μ l/reaction mixture) was achieved with 1.25 μ M random hexamer primers (Roche), 250 μ M each deoxynucleoside triphosphate (dNTP), 10 mM dithiothreitol (DTT), 20 U of RNase inhibitor, 2.5 mM $MgCl_2$, 200 U of MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega) in 30 mM Tris and 20 mM KCl (pH 8.3), and 2 μ g of sample RNA.

PCR was performed with gene-specific primers for the pyruvate-ferredoxin (Fd) oxidoreductase D (*pfoD*) gene (forward/reverse primer pair 5'-TCTCCGTTCTTGATCGTTC-3'/5'-TGTTGTGCGAAGACAGCCTG-3' [GenBank accession number [HQ657201](#)]) and the *T. vaginalis* [Fe]-hydrogenase A (Tvhya) gene (forward/reverse primer pair 5'-ATTATGTCATGCACACGAAA-3'/5'-ACACCACGATTGCACAAAA-3' [GenBank accession number [U19897](#)]). A parallel PCR with primers for the β -tubulin (*tub2*) gene (forward/reverse primer pair 5'-TACTCCATCGTCCCACTCC-3'/5'-CCGGACATAACCATGGAAAC-3' [GenBank accession number [L05469](#)]) was used as a reference gene for quantitative real-time reverse transcriptase PCR (RT-qPCR). Primer sets were designed and optimized by using the Primer 3Plus program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>), based on default parameters. PCR mixtures (20 μ l) contained 10 μ l Maxima SYBR green qPCR Master Mix (Thermo Scientific), the primer pair at 300 nM, 1 μ l of cDNA, and RNase-DNase-free water. PCR mixtures were subjected to 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. This was followed by melting-curve analysis at 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s. The specificity and size of PCR products for each gene were confirmed by gel electrophoresis. All PCRs were performed in an Eco real-time PCR system (Illumina). Relative quantification of gene expression was determined by the $2^{-\Delta\Delta C_q}$ method (29) by using software conforming to MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (30).

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5% linear gel (31, 32). The CE samples were reduced and denatured by incubation for 5 min at 100°C with 62 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 0.02 M DTT. Electrophoresis was performed in a minivertical electrophoresis system (Hoefer, USA) for 45 min at a constant voltage of 200 V in electrode buffer containing 25 mM Tris, 190 mM glycine, and 1% SDS (pH 8.3).

Western blot analysis was performed as previously described, with minor modifications (33). Following electrophoresis, reduced CE were immunoblotted at 15 V for 35 min onto Immobilon-P transfer membranes (0.45 μ m; Millipore, USA) in a Trans-Blot SD transfer cell (Bio-Rad, USA) with electrode buffer containing 48 mM Tris, 29 mM glycine, 0.037% SDS, and 20% methanol (pH 9.2). Membranes were washed with Tris-buffered saline (TBS) (50 mM Tris, 0.15 M NaCl [pH 7.4]), stained with Ponceau S (to verify protein transfer), transfer blocked for 2 h at room temperature with TBS containing 0.2% Tween 20 and 5% nonfat dry milk, washed in TBS, incubated for 1 h with a 1:500 dilution of rabbit polyclonal antibody raised against the highly conserved sequence at amino acids 342 to 641 mapping at the C terminus of heat shock protein 70 (Hsp70) of human origin (Santa Cruz Biotechnology, USA), and finally incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (dilution, 1:2,000; Dakopatts). Immunodetection of Hsp70 proteins was carried out with an enhanced luminol-based chemiluminescent substrate for detection of HRP (ECL Western blotting substrate; Thermo Scientific, USA), according to the manufacturer's instructions. Digital images were obtained after exposing the membrane to a chemiluminescent imaging system (FC2; FlorChem, USA) equipped with automatic image capture software. Finally, the bands in the digitized images were quantified by densitometry analysis (ImageMaster Total Lab, version 2.00; Amersham Pharmacia Biotech).

Data presentation and statistical analysis. The results are expressed as means $\pm$ standard errors of the means (SEM). The data were examined by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test for multiple comparisons, and differences were considered significant at an α value of 0.05. The concentration of drugs that caused a 50% inhibition of the response (IC_{50}) was estimated by nonlinear regression analysis (GraphPad Prism software; GraphPad, San Diego, CA, USA) from the concentration-response curves obtained in each case (34).

RESULTS

Effect of RESV on *in vitro* growth of *T. vaginalis*. The effects of RESV (0, 25, 50, and 100 μ M) and MDZ (0 and 6.25 to 100 μ M) on the *in vitro* growth kinetics of *T. vaginalis* trophozoites were compared (Fig. 1). At all concentrations tested, RESV caused a

FIG 2 Tvhyd activity in CE from *T. vaginalis* strain Tv1. Tvhyd activity was measured by monitoring the oxidation of reduced methyl viologen (MV) in CE from *T. vaginalis* treated with RESV at 25 and 50 μM (A) and MDZ at 3.125 and 6.25 μM (B). Histogram bars show means $\pm$ standard errors ($n = 5$). Asterisks indicate statistically significant differences between the treated groups and the untreated controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C) Tvhyd activity in a native stained gel after PAGE of *T. vaginalis* CE. Lanes containing CE were incubated in the presence of 12.5 μM MDZ or 100 μM RESV or without drugs (lane 0). Lane 0 shows a stained band with a molecular mass of approximately 120 kDa (arrow) that disappears in the lanes incubated with both drugs. Mw, molecular weight markers (in thousands).

significant decrease in the *in vitro* growth, which was detected on day 1 of culture. However, while concentrations of 50 and 100 μM significantly decreased the number of trophozoites (relative to the control) and appeared to be cytotoxic, the lowest concentration of RESV used (25 μM) significantly inhibited cell growth, indicating a cytostatic activity (Fig. 1A). The kinetics of antiparasitic activity of MDZ against *T. vaginalis* was very similar to that of RESV, although the former appeared to be slightly more toxic. At concentrations above 6.25 μM, MDZ produced a decrease in the number of parasites in culture from 24 h, thus displaying cytotoxic activity; at the lowest concentration tested, the drug produced cytostatic activity (Fig. 1B). The mean inhibition values (IC_{50}) obtained for RESV were 32 μM on day 1 and 25 μM on day 2, and the mean IC_{50} s for MDZ were 3.2 μM on day 1 and 4.25 μM on day 2 of culture.

Effect of RESV on Tvhyd activity. The specific activity of Tvhyd was quantified by a spectrophotometric assay, and the effect of RESV (25 and 50 μM) on enzyme activity was determined. The effect of MDZ (3.125 and 6.25 μM) on Tvhyd activity was also determined. At the concentrations tested, both RESV and MDZ produced a significant dose-dependent decrease in Tvhyd activity, with mean IC_{50} s of 35.6 and 3.2 μM, respectively (Fig. 2A and B).

In a parallel experiment, the effect of RESV and MDZ (100 and 12.5 μM, respectively) on Tvhyd activity was also tested qualitatively on native PAGE gels (Fig. 2C). The bands indicating Tvhyd

activity were detected when the CE were applied onto PAGE gels, and the resulting gels were stained with MV. Native PAGE revealed the presence of a single band of 120 kDa, which was stained when CE of untreated trophozoites were incubated anaerobically with MV and H_2 gas. However, the band disappeared when the gels containing the CE were incubated in the presence of 12.5 μM MDZ or 100 μM RESV (Fig. 2C).

Effect of RESV and MDZ on energetic state of the hydrogenosome. The effect of RESV and MDZ on the energetic state of the hydrogenosome of *T. vaginalis* was analyzed by using cell-permeant MitoTracker Deep Red probes. The *T. vaginalis* trophozoites incubated with the probe were fluorescent, often showing intracytoplasmic fluorescent granules of shape and size compatible with hydrogenosomes (Fig. 3B).

Fluorescence levels of trophozoites incubated with MitoTracker Deep Red FM probe were quantified by fluorometry. Trophozoites incubated with RESV (50 and 100 μM) or MDZ (5.12 to 6.25 μM) exhibited significantly higher, dose-dependent fluorescence than untreated controls (Fig. 3A).

Effect of RESV and MDZ on ROS production and on the $\Delta\psi_m$ of the hydrogenosome. *T. vaginalis* trophozoites were initially incubated under microaerobic conditions, and intracellular ROS production was determined by using the fluorescent probe carboxy-DCFDA in a fluorometric assay. Addition of RESV (25, 50, and 100 μM) or MDZ (3.125, 5.12, and 6.25 μM) did not

FIG 3 (A) Effect of RESV at 50 and 100 μM and MDZ at 6.25 and 12.5 μM on the energetic state of the *T. vaginalis* hydrogenosome determined by using the cell-permeant MitoTracker probe MitoTracker Deep Red FM. Results are expressed in arbitrary units of fluorescence, and the bars show means $\pm$ standard errors ($n = 5$). Asterisks indicate statistically significant differences with respect to control values (*, $P < 0.01$). (B) Detection of *T. vaginalis* hydrogenosomes by fluorescence microscopy in cells stained with MitoTracker Red. Hydrogenosomes appear intensely stained red (arrows).

significantly affect intracellular ROS production (Fig. 4A). In a second experiment, the fluorescent probe JC-1 was used to analyze the effect of RESV on the $\Delta\psi\text{m}$ in treated and untreated *T. vaginalis* trophozoites after incubation for 2 h with the drugs in MDM. In this case, there was a significant dose-dependent decrease in the $\Delta\psi\text{m}$ in the trophozoites incubated for 2 h with MDZ or RESV (Fig. 4B).

Influence of RESV on hydrogenosome enzyme gene expression. The effect of RESV and MDZ on the expression of two key genes within the hydrogenosome metabolism of *T. vaginalis* was evaluated: the TvhydA gene and the gene encoding the enzyme pyruvate-ferredoxin oxidoreductase (PFO), responsible for pyruvate oxidation (*pfoD* gene). Although RESV (100 μM) did not affect TvhydA gene expression, MDZ (3.125 and 6.25 μM) had a significant dose-dependent effect on expression of this gene (Fig. 5). However, treatment with RESV (100 μM) increased *pfoD* gene expression, and MDZ (3.125 and 6.25 μM) also significantly increased the levels of expression of this gene (Fig. 5).

Effect of RESV on Hsp70 expression. Finally, the effect of RESV and MDZ on Hsp70 protein expression in *T. vaginalis* trophozoites was analyzed by Western blotting. The results indicated a dose-dependent increase in the protein expression level of these chaperones in the trophozoites of *T. vaginalis* treated with RESV (50 to 100 μM) or MDZ (3.125 and 6.25 μM) (Fig. 6).

DISCUSSION

The 5-nitroimidazole drugs, of which MDZ is the most commonly prescribed, are the only effective drugs approved for treatment of trichomoniasis (6, 35). MDZ is considered to be cost-effective because it is inexpensive, is generally efficient in eliminating *T. vaginalis* infection, has favorable pharmacokinetic and pharmacodynamic properties, and exerts minor adverse effects (8). Resistance to MDZ is frequently reported, and cross-resistance among the family of 5-nitroimidazole drugs is common, leaving no alternative treatments so that some cases remain unresolved (35). Alternative treatments for trichomoniasis are also needed for individuals who are allergic to MDZ (36, 37). The reliance on a single class of antimicrobial drugs for treatment of *T. vaginalis* infections

may also increase the emergence of resistance, and studies of novel therapeutic options for treatment and prevention of refractory trichomoniasis are therefore essential (5). This study demonstrates that *in vitro* treatment with RESV significantly inhibits growth of *T. vaginalis* and displays a very similar kinetic antiparasitic activity to that produced by the reference drug MDZ although with slightly lower toxicity to the parasite. Mechanistic studies of cells *in vitro* have almost invariably used concentrations of RESV in the range of 10^{-5} to 10^{-4} M to establish the therapeutic efficacy of this compound (38). Some studies have analyzed the *in vitro* antitrichomonal activity of some natural extracts from medicinal plants, which displayed good antitrichomonal activity, with IC_{50} s ranging between 5.6 and 8.0 $\mu\text{g}/\text{ml}$ (39–43). These values are similar to those obtained for RESV in this study but much higher than those obtained for MDZ, the antiprotozoal drug used as a positive control, which in the present study yielded a slightly higher value than in other studies (44).

The hydrogenosome, which is a spherical or slightly elongated structure with a diameter of 0.5 to 1.0 μm , is usually associated with cytoskeletal structures such as the axostyle and costa in trichomonads (45). This unusual organelle, which is found in *T. vaginalis*, is an anaerobic form of mitochondrion (46, 47) and produces H_2 during ATP synthesis (46). In *T. vaginalis*, fermentative energy metabolism relies on the highly oxygen-sensitive enzyme pyruvate-ferredoxin oxidoreductase (PFO), which is an iron-sulfur protein that converts pyruvate to acetyl coenzyme A (acetyl-CoA) (48), and Tvhyd (49). Hydrogenases (cytochrome c_3 oxidoreductase [EC 1.18.99.1]) are classified, on the basis of the metal content of their dinuclear catalytic centers, as [NiFe], [Fe], or [NiFeSe] hydrogenases (50). There is evidence that Tvhyd belongs to the [Fe]-hydrogenases, which serve as terminal electron acceptors, evolving molecular hydrogen (51, 52). The present results demonstrate that both MDZ and RESV cause dose-dependent inhibition of H_2 production by the *T. vaginalis* hydrogenosome, as determined by measurement of specific activity by photochemically reduced MV.

Two closely related Tvhyd genes from *T. vaginalis*, TvhydA and TvhydB, have previously been characterized, and one of these

FIG 4 (A) Intracellular ROS production by *T. vaginalis* trophozoites incubated in the presence of RESV at 25, 50, and 100 μM and MDZ at 3.125, 6.25, and 12.5 μM. Prooxidant levels were measured by using the oxidation-sensitive fluorescent probe 5(6)-carboxy-2',7'-dichlorofluorescein diacetate. (B) Effect of RESV at 25, 50, and 100 mM and MDZ at 3.125, 6.25, and 12.5 μM on membrane potential in the *T. vaginalis* hydrogenosome, quantified by using the lipophilic cationic probe JC-1. The results are expressed in arbitrary units of fluorescence (AU). Histogram bars show means $\pm$ standard errors ($n = 5$). Asterisks indicate statistically significant differences between the treated groups and the untreated controls (*, $P < 0.05$; **, $P < 0.01$).

contains a 50-kDa protein (53). Other authors subsequently isolated a gene encoding a putative 64-kDa-like [Fe]-hydrogenase from *T. vaginalis* (54). We analyzed the presence of Tvhyd in CEs by PAGE under native conditions, and we found a single protein band of about 120 kDa. Most of the Tvhyd proteins characterized so far consisted of at least two subunits (55), which may indicate that Tvhyd has a dimeric structure, which is consistent with the sizes predicted by genetic analysis (54). As in the spectrophotometric assay, native PAGE showed that both MDZ and RESV inhibit hydrogenase activity. Most studies have established that the

antiparasitic mechanism of MDZ is related to the generation of cytotoxic anion radicals (nitro radicals), which bind transiently to DNA, disrupt the DNA, and cause cell death (4, 25). It has been established that the electrons required for MDZ activation are released from pyruvate and malate by the activity of the PFO enzyme and are transferred to the drug by a low-redox-potential carrier, ferredoxin (Fd), which is reduced to its cytotoxic nitro radical anion (25). In the hydrogenosome, electrons released in the PFO reaction are accepted by Fd, which is subsequently reoxidized by Tvhyd, and in this reaction, electrons are coupled to

FIG 5 Expression level of ferredoxin oxidoreductase (PfoD) and hydrogenase (TvhydA) genes after exposure to RESV (50 to 100 μ M) and MDZ (3.125 to 6.25 μ M), measured by RT-qPCR, using the β -tubulin (btub2) gene as a reference for gene expression normalization. Analysis of relative gene expression levels was done by the $2^{-\Delta\Delta Ct}$ method, and each vertical bar represents the mean $\pm$ standard error. Asterisks indicate statistically significant differences between the treated groups and the untreated controls (*, $P < 0.05$; **, $P < 0.01$).

protons to form H_2 (56). MDZ acts as an electron sink in *T. vaginalis* by capturing the electrons generated by PFO, which are then transported by Fe [2Fe-2S] to the drug and not to their natural acceptor, Tvhyd. MDZ thus effectively competes for electrons with Tvhyd, and consequently, activation of the drug is reflected by reduced H_2 production by hydrogenosomes (57). Several mechanisms are potentially responsible for the RESV-induced inhibition of H_2 production in hydrogenosomes: (i) competition with Tvhyd for electrons (like MDZ), (ii) inhibition of PFO activity and prevention of the generation of electrons that are transferred to Tvhyd, (iii) inhibition of the transport of electrons from Fd, and (iv) direct inhibition of Tvhyd. Because RESV and MDZ

inhibited H_2 production in the band corresponding to Tvhyd in native PAGE gels, the effects of RESV on H_2 production must involve competition for electrons generated by PFO or enzyme inhibition. Although it is known that RESV may act as a radical-scavenging antioxidant via its chemical repair free radicals (56), there is no evidence for any ability to compete for electrons, as described previously for MDZ (58). Therefore, in view of the present results, it could be inferred that RESV acts primarily as an inhibitor of Tvhyd. It was suggested previously that iron may regulate hydrogenosomal activity through hydrogenosomal enzyme expression and $\Delta\psi_m$ (59). Therefore, it is possible that the inhibitory effect of RESV on the Tvhyd enzyme may involve Fe transport, as it was shown previously that RESV blocks Fe transport and can act as an enzyme inhibitor (60).

MitoTracker Deep Red FM is a far-red-fluorescent dye (excitation/emission $\sim 644/665$ nm) that stains mitochondria and hydrogenosomes in live cells and can be used for *in vivo* determination of the mass and localization of the hydrogenosome in *T. vaginalis* (61, 62). We noted that both RESV and MDZ significantly increased the fluorescence emitted by the parasite hydrogenosomes incubated with MitoTracker Deep Red FM, suggesting that both compounds induce changes in hydrogenosomal metabolism in *T. vaginalis* trophozoites. This increase in fluorescence may be related to the induction of oxidative stress, a phenomenon that has also been detected in other parasites treated with RESV (63). Thus, our previous studies on parasitic ciliates indicated that treatment with RESV yielded a significant increase in intracellular ROS production and that oxidative damage may preferentially affect the stability and function of enzymes containing iron-sulfur clusters, such as complexes II and III (63–65). However, the present results indicate that either RESV or MDZ did not significantly increase ROS production in *T. vaginalis*. *T. vaginalis* has a microaerophilic life-style and uses redox antioxidant systems to counter the detrimental effects of oxygen and express a wide range of genes encoding defense molecules, including superoxide dis-

FIG 6 (A) Western blot analysis of CE of *T. vaginalis* incubated with anti-Hsp70 antibodies. *T. vaginalis* trophozoites were incubated in the presence of RESV at 50 and 100 μ M and MDZ at 3.125 and 6.25 μ M. —, control without the addition of anti-Hsp70 antibody. Mw, molecular weight marker (in thousands). (B) Densitometric analysis of the immunostained CE from panel A (see Materials and Methods for a description of band quantification).

mutases, thioredoxin reductases, peroxiredoxins, and rubrerythrins (4), which may eliminate the small amount of ROS produced.

JC-1 is a fluorescent lipophilic and cationic probe that can be used to determine the mitochondrial and hydrogenosomal $\Delta\psi_m$ (28, 66). Use of the JC-1 probe revealed that RESV and MDZ induced a loss of $\Delta\psi_m$, suggesting that these agents also produced a metabolic perturbation of the hydrogenosome. $\Delta\psi_m$ may be altered by deregulation of intracellular ionic charges (e.g., H^+) caused by alterations in Tvhyd activity. In ciliate parasites, RESV also generates a collapse and dysfunction of mitochondrial $\Delta\psi_m$ accompanied by a significant increase in intracellular Ca^{2+} levels (65).

Several studies have correlated MDZ resistance with deregulation of hydrogenosomal enzyme gene expression (67). Thus, some studies have shown a significant reduction in PFO and Tvhyd transcription levels in strains of *T. vaginalis* that are resistant to MDZ (68). The present results indicate that MDZ has an inductive effect on expression of the PFO gene (like RESV) at the highest dose used and also on the expression of the gene encoding the Tvhyd enzyme in our strain of *T. vaginalis*. Therefore, the increased expression levels of both enzymes may be related to the maintenance of homeostasis in the hydrogenosome in parasites treated with both compounds.

The results obtained in the present study suggest that RESV and MDZ caused a high degree of bioenergetic stress in treated parasites. Like mitochondria, hydrogenosomes of *T. vaginalis* contain the heat shock proteins Hsp70, Hsp60, and Hsp10 (69). The primary function of the chaperone 70 families is to maintain mitochondrial homeostasis and quality control (70). Overexpression of Hsp70 during treatment may be related to an attempt by the parasite to aid hydrogenosomal survival by protecting against induced damage caused by the drugs.

In conclusion, this study confirms the *in vitro* antitrichomonal activity of RESV, demonstrating that the antiparasitic mechanism of this polyphenol occurs through induction of hydrogenosomal metabolism alteration. This effect on trichomonal energy metabolism leads to a profound dysfunction of the hydrogenosome, which has deleterious effects on the parasite.

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