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O_2-Dependent Efficacy of Novel Piperidine- and Piperazine-Based Chalcones against the Human Parasite *Giardia intestinalis*

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Giardia intestinalis **is the most frequent protozoan agent of intestinal diseases worldwide. Though commonly regarded as an anaerobic pathogen, it preferentially colonizes the fairly oxygen-rich mucosa of the proximal small intestine. Therefore, when testing new potential antigiardial drugs, O_2 should be taken into account, since it also reduces the efficacy of metronidazole, the gold standard drug against giardiasis. In this study, 46 novel chalcones were synthesized by microwave-assisted Claisen-Schmidt condensation, purified, characterized by high-resolution mass spectrometry, ^1H and ^{13}C nuclear magnetic resonance, and infrared spectroscopy, and tested for their toxicity against *G. intestinalis* under standard anaerobic conditions. As a novel approach, compounds showing antigiardial activity under anaerobiosis were also assayed under microaerobic conditions, and their selectivity against parasitic cells was assessed in a counterscreen on human epithelial colorectal adenocarcinoma cells. Among the tested compounds, three [30(a), 31(e), and 33] were more effective in the presence of O_2 than under anaerobic conditions and killed the parasite 2 to 4 times more efficiently than metronidazole under anaerobiosis. Two of them [30(a) and 31(e)] proved to be selective against parasitic cells, thus representing potential candidates for the design of novel antigiardial drugs. This study highlights the importance of testing new potential antigiardial agents not only under anaerobic conditions but also at low, more physiological O_2 concentrations.**

Giardiasis is a worldwide waterborne intestinal parasitic disease, caused by the amitochondriate protist *Giardia intestinalis* (1, 2). This flagellated unicellular eukaryote, first discovered by the Dutch microscopist Antony van Leeuwenhoek in 1681, has a relatively simple life cycle. It spreads in the environment as a stable, highly infectious cyst; once ingested by the host, it reaches the stomach lumen and develops into its vegetative form, the trophozoite, which attaches to the intestinal epithelium and starts proliferating in the proximal small intestine, eventually causing the disease. Here, this microaerobic parasite must survive exposure to O_2, as well as nitric oxide (NO) and related reactive species, most likely through the intervention of a battery of detoxifying enzymes (3–7). Following encystation, the parasite is finally expelled back to the environment, ready to infect other hosts.

The most important clinical symptom of giardiasis is diarrhea, often severe and protracted, with malabsorption, dehydration, weight loss, failure to thrive, cognitive impairment in children, and chronic fatigue in adults (8, 9). The incidence of the disease is higher for 1- to 9-year-old children and 30- to 39-year-old adults (2). There are several medications approved or commonly used for the treatment of giardiasis to eradicate the parasite from the host intestine. Metronidazole (MTZ), tinidazole, furazolidone, paromomycin, and nitazoxanide are frequently used as antigiardial drugs (10, 11). MTZ, the drug of choice, is a 5-nitroimidazole prodrug that is intracellularly redox activated to a nitroradical cytotoxic form by reduction of the nitro moiety (12). Relevant to this study, the active drug is in turn converted back to the nontoxic parent compound upon reaction with O_2 (12). Many problems are associated with the use of antigiardial agents, including treatment failures, unpleasant side effects, activity against normal intestinal flora, possible carcinogenicity, and parasite resistance (10, 13). The development of new drugs against *G. intestinalis* is therefore a matter of concern (11).

Compared to other intestinal compartments, the epithelium of the proximal small intestine, where *Giardia* trophozoites adhere with their ventral disks causing the disease, is a fairly aerobic environment, where O_2 is delivered mostly by the submucosal vascular network (14). The presence of O_2 there can drastically affect the killing efficacy of drugs specifically designed against the parasite. This notwithstanding, except for MTZ (15) and menadione (16) inactivation, the O_2 effect on the action of new potential antigiardial compounds is usually neglected in the literature, as drug susceptibility assays are mostly performed under anaerobic conditions (17–24).

Chalcones, 1,3-diphenyl-2-propen-1-one derivatives belonging to the flavonoid family, are open-chain unsaturated carbonyl systems in which two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl framework. Recently, compounds with a chalcone (1,3-diaryl-2-propen-1-one) moiety have been reported to exhibit diverse pharmacological effects, including antimalarial (25), antiviral

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(26), antibacterial (27), antituberculosis (28), antifungal (29), anticancer (30), antileishmanial (31), anti-inflammatory (32), and even antigiardial (33) effects. A number of chalcone derivatives have also been found to inhibit several important enzymes in cellular systems, including protein tyrosinase (34), malarial aspartic acid protease, plasmepsin II (35), and malarial cysteine protease falcipain-2 (36). Numerous reports suggest the value of synthetic chalcones as key intermediates in the synthesis of different heterocyclic scaffolds, pyrimidines (37) and pyrazoles (38, 39).

Appreciation of the broad pharmacological spectrum of chalcones led us to design and synthesize new piperidine- and piperazine-based chalcone derivatives as attractive drug scaffolds. For their biological activities, heterocycles with a nitrogen atom are undoubtedly an important class of highly applicable bioactive molecules of pharmaceutical interest, used as a key structural motif for the synthesis of various medicines.

In the present study, two libraries of novel chalcone derivatives, for a total of 46 compounds, were synthesized, purified, characterized, and tested for their toxicity against *G. intestinalis* and human epithelial colorectal adenocarcinoma (Caco-2) cells as a control to assess their selectivity. Active compounds were comparatively assayed under both anaerobic and more physiological microaerobic conditions. This novel approach allowed us to identify two chalcone derivatives that under microaerobic conditions are able to kill *Giardia* trophozoites selectively and more efficiently than MTZ.

MATERIALS AND METHODS

Materials. All chemicals used in the synthesis of chalcones were purchased from Sigma-Aldrich and Fluka. MTZ, ATP, penicillin-streptomycin, bovine calf serum, bovine bile, and the chemicals for the Diamond TYI-S-33 medium used for *Giardia* cell cultures were from Sigma-Aldrich. Fetal bovine serum, glutamine, nonessential amino acids, trypsin-EDTA, and Eagle's minimum essential medium (EMEM) were purchased from Gibco (Life Technologies). Other chemicals and solvents purchased locally were of analytical grade. Caco-2 cells (ATCC HTB-37) were purchased from Sigma-Aldrich. Incubation bags for anaerobiosis (Anaerocult A minisystem) and microaerobiosis (Anaerocult C minisystem) were from Merck. Sterile 96-well white clear-bottom plates were purchased from PerkinElmer. The ATP one-step luminescence assay systems for microbial (BacTiter-Glo) and human (ATPlite) cells were from Promega and PerkinElmer, respectively.

Synthesis and characterization of chalcones. (i) Chemical methods. The homogeneity and purity of all the products were analyzed by thin-layer chromatography (TLC) on alumina-coated plates (Merck). Product samples in methanol (MeOH) were loaded on TLC plates and developed in $CHCl_3$-MeOH (9.7:0.3, vol/vol). When slight impurities were detected by iodine vapor-UV light visualization, compounds were further purified by chromatography on silica gel columns (100 to 200 mesh size; CDH), using petroleum ether-ethyl acetate (3:2, vol/vol) as the eluent. Microwave-assisted synthesis of chalcones was carried out in a CEM Discover microwave instrument. Melting points were determined in open glass capillary tubes on a Buchi M-560 instrument and are uncorrected. Infrared (IR) spectra were recorded in KBr medium using a Perkin-Elmer Fourier transform-IR spectrometer, whereas ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ medium on a JNM ECX-400P (JEOL, USA) spectrometer with tetramethylsilane as internal reference. IR and NMR spectra were recorded at the Department of Chemistry, University of Delhi, Delhi, India. Absorption frequencies (ν) are expressed in cm^{-1}, chemical shifts are expressed in ppm (δ-scale), and coupling constants (J) are expressed in Hz. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). High-resolution mass spectroscopy (HRMS) data were collected on a JEOL JMS-SX-102A spectrometer at the Institute for Chemistry and Biochemistry, Freie Universität, Berlin, Germany.

TABLE 1 Chemical identities, reaction times, and purification yields of chalcones 30(a-w), 31(a-u), 32, and 33, together with their antigiardial activity measured under anaerobiosis

Compound	R_1	R_2	R_3	R_4	R_5	Time (min)	Yield (%)	IC_{50} (μM) for *Giardia intestinalis*
30a	H	H	H	H	H	15	84	12.3
30b	Cl	H	H	H	H	15	92	>100
30c	H	Cl	H	H	H	20	77	>100
30d	H	H	Cl	H	H	30	87	>100
30e	Cl	H	H	H	Cl	25	56	>100
30f	H	Cl	Cl	H	H	30	45	>100
30g	Br	H	H	H	H	25	70	>100
30h	H	Br	H	H	H	20	95	>100
30i	H	H	Br	H	H	30	98	>100
30j	F	H	H	H	H	30	87	>100
30k	H	F	H	H	H	15	63	>100
30l	H	H	F	H	H	20	87	>100
30m	H	F	F	H	H	30	95	>100
30n	H	CH_3	H	H	H	25	72	>100
30o	H	H	CH_3	H	H	25	91	>100
30p	H	H	CF_3	H	H	25	85	>100
30q	H	H	$N(CH_3)_2$	H	H	30	89	>100
30r	H	H	$N(C_2H_5)_2$	H	H	25	59	>100
30s	H	H	OCH_3	H	H	30	91	>100
30t	OCH_3	H	H	OCH_3	H	25	89	>100
30u	H	OCH_3	OCH_3	H	H	25	88	>100
30v	OCH_3	H	OCH_3	H	OCH_3	20	81	>100
30w	H	OCH_3	OCH_3	OCH_3	H	25	57	>100
31a	H	H	H	H	H	20	90	>100
31b	Cl	H	H	H	H	25	70	>100
31c	H	Cl	H	H	H	25	48	>100
31d	H	H	Cl	H	H	20	53	>100
31e	Cl	H	H	H	Cl	25	70	21.0
31f	H	Cl	Cl	H	H	30	45	>100
31g	H	Br	H	H	H	20	85	>100
31h	H	H	Br	H	H	25	68	>100
31i	F	H	H	H	H	25	54	>100
31j	H	F	H	H	H	15	98	>100
31k	H	H	F	H	H	25	98	>100
31l	F	H	H	H	F	15	91	>100
31m	H	F	F	H	H	15	90	>100
31n	H	CH_3	H	H	H	20	77	>100
31o	H	H	CH_3	H	H	20	80	>100
31p	H	H	$N(CH_3)_2$	H	H	20	60	>100
31q	H	H	OCH_3	H	H	30	69	>100
31r	OCH_3	H	H	OCH_3	H	30	70	>100
31s	H	OCH_3	OCH_3	H	H	25	70	>100
31t	OCH_3	H	OCH_3	H	OCH_3	30	60	>100
31u	H	OCH_3	OCH_3	OCH_3	H	30	40	>100
32	H	H	H		H	25	88	>100
33	H	H	H		H	25	70	16.8

(ii) General procedure for the synthesis of intermediate compounds 4 and 5. To a well-stirred mixture of compound 1 (4-benzylpiperidine) (1 mmol) or compound 2 (4-benzylpiperazine) (1 mmol) and anhydrous K_2CO_3 (1.5 eq) in dimethyl sulfoxide (DMSO) (7 ml), compound 3 (4-fluoroacetophenone) (1 mmol) was added slowly. The reaction mixture was heated to 100°C for 18 h, and the progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was allowed to cool down to room temperature. The reaction mixture was then poured into vigorously stirred crushed ice, and the obtained precipitate was filtered, washed with water, and recrystallized from dry MeOH.

(iii) General procedure for the synthesis of chalcones 30(a-w), 31(a-u), 32, and 33. To a solution of aryl ketone (compound 4 or 5) (1 mmol) and aromatic aldehyde (compounds 6 to 29) (1 mmol) in dry MeOH (4 ml) taken in microwave vials, a catalytic amount of sodium hydroxide (1 pellet) was added, and the reaction mixture was irradiated by microwave radiation at 60°C for different time intervals (Table 1) at a power of 100 W. The reaction progress was monitored by TLC using MeOH-$CHCl_3$ (0.3:9.7, vol/vol) as the solvent. When the reaction was found to be complete, the reaction mixture was allowed to cool down to room temperature and then poured into ice-cooled water. The obtained yellowish precipitate was

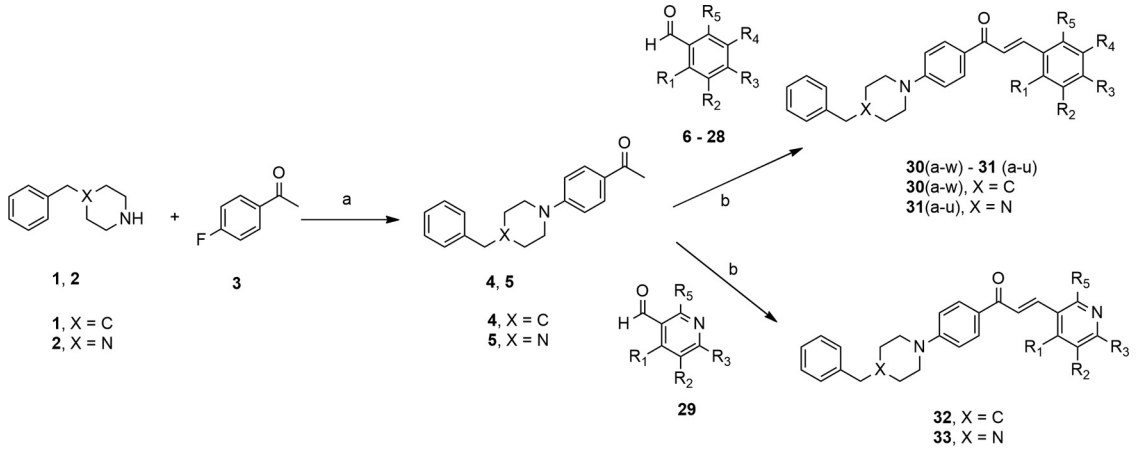

FIG 1 Synthetic route to compounds 30(a-w), 31(a-u), 32, and 33. (a) K_2CO_3, DMSO for 18 h, 100°C. (b) NaOH, MeOH, microwave, 100 W, 60°C, 15 to 30 min.

filtered and dried on vacuum. The compounds were finally purified by silica gel (100 to 200 mesh size) column chromatography, using ethyl acetate-petroleum ether (2:3 [vol/vol]) as the eluent. Prior to being tested in cell viability assays, all compounds were dissolved in DMSO to 7 to 8 mM.

***Giardia* culture.** Trophozoites of *G. intestinalis* strain WB clone C6 (ATCC no. 50803) were cultured axenically at 37°C in 25-cm^2 flasks containing Diamond's TYI-S-33 medium supplemented with 10% bovine calf serum, 1 mg/ml bovine bile, 0.1 g/liter streptomycin, and 100 U/ml penicillin. Typically, 50 ml medium was inoculated with 25×10^6 cells, and after 2 days, cells were harvested by chilling the flasks on ice for 30 min for drug susceptibility assays.

Antigiardial activity assay. The antigiardial activity assay was performed according to the procedure described in reference 40, using sterile 96-well white clear-bottom plates. In each well, 50 μl culture medium containing 0.4×10^6 cells/ml *Giardia* trophozoites was added to 50 μl medium containing either the compound to be tested, serially diluted from a stock solution in DMSO, or the same amount of DMSO as a control; this yielded a final density of 20,000 cells/100 μl in each well. Each drug concentration assay was performed at least in 12 replicates. MTZ was used as an internal positive control in the assay. The microtiter plates were then incubated at 37°C under anaerobic or microaerobic conditions, ensured by the Anaerocult A or the Anaerocult C minisystem (Merck), respectively. According to the manufacturer's instructions, the Anaerocult A minisystem produces anaerobic conditions within ~1 h, whereas the Anaerocult C minisystem generates microaerobic conditions (~5% O_2) within 24 h. Following 48 h of incubation with the compound to be tested, 100 μl of the BacTiter-Glo microbial cell viability assay system reagent (Promega) was added to each well for one-step lysis and ATP level detection. Plates were then incubated at room temperature for 15 min, and ATP levels were finally detected by luminescence on a plate reader (Wallac Victor^3 1420 multilabel counter; PerkinElmer).

Cytotoxicity assay on Caco-2 cells. Caco-2 cells were grown in 25-cm^2 flasks in Eagle's minimum essential medium (EMEM) supplemented with 1% (vol/vol) nonessential amino acids, 2 mM glutamine, 5% (vol/vol) fetal bovine serum, 0.1 g/liter streptomycin, and 100 U/ml penicillin. Prior to each assay, cells were detached with 0.5% trypsin-EDTA and seeded in sterile 96-well white clear-bottom plates at the same density of *Giardia* cells and at increasing concentrations of the compound to be tested, as described for *Giardia* trophozoites. The assay was carried out exactly as reported for *Giardia* cells, except that the plates were incubated (still at 37°C) at atmospheric O_2 level, 5% CO_2, and 95% humidity. Each drug concentration assay was performed in at least six replicates. MTZ was used as an internal negative control in the assay. Following 48 h of incubation with each compound, according to the manufacturer's instructions, 100 μl of the ATPlite luminescence assay system (PerkinElmer) was added to each well for one-step lysis and ATP level detection by luminescence.

Determination of IC_{50} and SI. Luminometric data were calibrated using ATP standard curves and normalized to the ATP level measured in control DMSO-treated cells (taken as 100%). For each active compound, the measured ATP level percentage was plotted as a function of the compound concentration and the half-maximal inhibitory concentration (IC_{50}) was obtained by fitting the resulting titration profile to the Hill equation (41). The selectivity index (SI) of the compounds was then calculated as the ratio between the IC_{50} measured on human cells and the value determined on *Giardia* cells ($SI = IC_{50,Caco\text{-}2}/IC_{50,Giardia}$).

RESULTS

Synthesis of chalcones. The synthetic route to novel chalcones 30(a-w), 31(a-u), 32, and 33, based on previously described Claisen-Schmidt condensation (36), is outlined in Fig. 1. Intermediate compounds 4 and 5 were synthesized with 64% and 86% yield, respectively, by nucleophilic substitution of commercially available starting material 1 and 2 with 4-fluoroacetophenone (compound 3), in the presence of anhydrous K_2CO_3 in DMSO, at 100°C. As shown in Fig. 1, compounds 30(a-w), 31(a-u), 32, and 33 were obtained under 100-W microwave irradiation (for distinct time intervals, as reported in Table 1) of intermediate compound 4 or 5 in the presence of commercially available aromatic aldehydes 6 to 29, in the presence of NaOH in dry MeOH, at 60°C. After purification, not always optimized, the yield of products 30(a-w), 31(a-u), 32, and 33 ranged from 40% to 98%.

The abovementioned experimental conditions were found to be optimal, as organic weak bases such as triethylamine and piperidine generally gave lower yields. Sodium methoxide did not work as well as solid sodium hydroxide pellet. To ensure formation of the solid products, a minimal amount of MeOH was used. If the starting materials were found to be insoluble in MeOH, either tetrahydrofuran or 1,4-dioxane was used as a cosolvent.

Each of the synthesized chalcones was characterized by HRMS, ^1H and ^{13}C NMR, and IR spectroscopy, and relevant data are reported in the supplemental material.

Antigiardial activity in anaerobiosis. The newly synthesized compounds were first tested for their antigiardial activity under

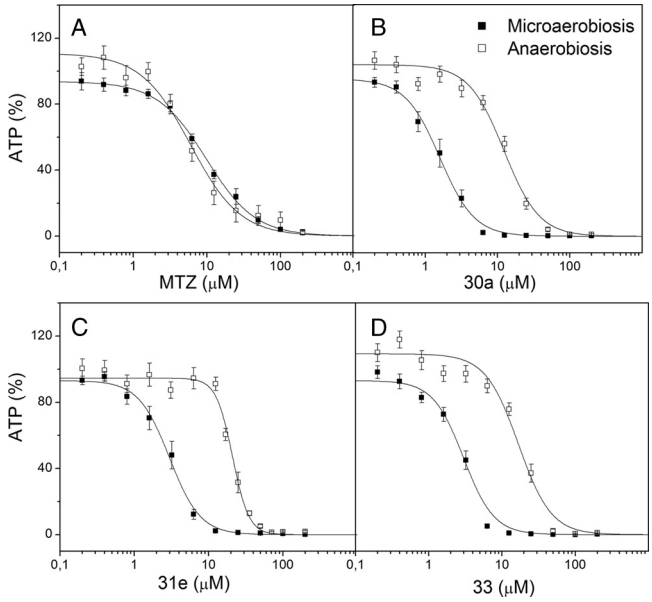

FIG 2 Dose-response curves of MTZ and the newly synthesized chalcone derivatives 30(a), 31(e), and 33 on *Giardia* trophozoites cultured in anaerobiosis (open squares) or in microaerobiosis (filled squares). Data are expressed as means ± standard errors ($n \geq 12$).

anaerobic conditions. According to reference 40, the susceptibility of *Giardia* cells to increasing concentrations of each compound was assessed by performing a cell viability assay based on ATP level determination by luminescence. Dose-response curves were obtained after 48 h of incubation with each compound and compared to data collected under identical conditions with MTZ, the drug of choice for treatment of giardiasis. All compounds were dissolved in DMSO. Thus, toxicity of DMSO on *Giardia* cells was also evaluated: DMSO at concentrations of ≤2% (vol/vol) decreased cell viability by up to 10% (not shown). Consistent with data reported in the literature (17), under the experimental conditions of the assay, MTZ effectively killed the parasites with an IC_{50} of 6.0 μM (Fig. 2A). Among the 46 synthetic compounds tested, only three showed a significant antigiardial activity, compounds 30(a), 31(e), and 33, with IC_{50}s equal to 12.3, 21.0, and 16.8 μM, respectively (Fig. 2B to D, open symbols). All other compounds caused either no effect or only a small decrease in *Giardia* viability at concentrations of ≥100 μM (Table 1).

Oxygen-dependent toxicity of hit compounds. The three active compounds were tested for their toxicity against *Giardia* trophozoites under microaerobic conditions (see Materials and Methods for details). In the presence of O_2, the three compounds showed a 5- to 7-fold-greater killing efficacy than under anaerobic conditions and proved to be more active (at least 3-fold) than MTZ, as assayed under the same microaerobic conditions. The IC_{50} of compound 30(a) dropped to 1.6 μM (Fig. 2B) and that of compounds 31(e) and 33 dropped to 3.0 μM (Fig. 2C and D). In contrast, the IC_{50} of MTZ rose by ~1.5-fold to 9.8 μM (Fig. 2A), making the results on hit compounds even more intriguing. A comparison of the IC_{50}s measured under microaerobic conditions and under anaerobic conditions is reported in Table 2.

Cytotoxicity on Caco-2 cells. In order to assess the selectivity of compounds 30(a), 31(e), and 33 against *Giardia*, their toxicity toward a mammalian cell line, Caco-2 cells, was tested after 48 h of incubation. In the assay, DMSO only slightly affected cell viability (up to 10% at ≤2% [vol/vol]; not shown). As shown in Fig. 3, analysis of the titration profiles measured for compounds 30(a), 31(e), and 33 yielded IC_{50}s equal to 40.5, 46.6, and 19.3 μM, respectively, whereas as expected MTZ had no effect on cell viability up to 200 μM. These results allowed us to calculate the selectivity index (SI) for the three synthetic compounds (Table 2), defined as the ratio between the IC_{50}s measured on human cells and those measured on parasitic cells. Interestingly, an SI greater than 10, indicative of a preferential toxicity against parasitic cells (compared to human cells), was obtained for compounds 30(a) and 31(e) from the IC_{50}s measured on *Giardia* cells under microaerobic conditions.

TABLE 2 Effect of O_2 on the antigiardial efficacy of active chalcones compared to metronidazole

Compound	IC_{50} (μM)			SI	
	G. intestinalis				
	Anaerobiosis	Microaerobiosis	Caco-2 cells	Anaerobiosis	Microaerobiosis
30(a)	12.3	1.6	40.5	3.3	25.3
31(e)	21.0	3.0	46.6	2.2	15.6
33	16.8	3.0	19.3	1.2	6.4
MTZ	6.0	9.8	>200		

DISCUSSION

Though being commonly regarded as an "anaerobic protozoon" (2) and studied as such in the laboratory, the human parasite *G. intestinalis in vivo* preferentially colonizes a fairly aerobic environment, the mucosa of the proximal small intestine. Here, O_2 is supplied both with swallowed air (42) and by the fine microcirculatory vascular network perfusing the intestinal submucosa (43). O_2 level in the gut varies among individuals and is also affected by the intestinal microbiota. Moreover, available O_2 in the intestine

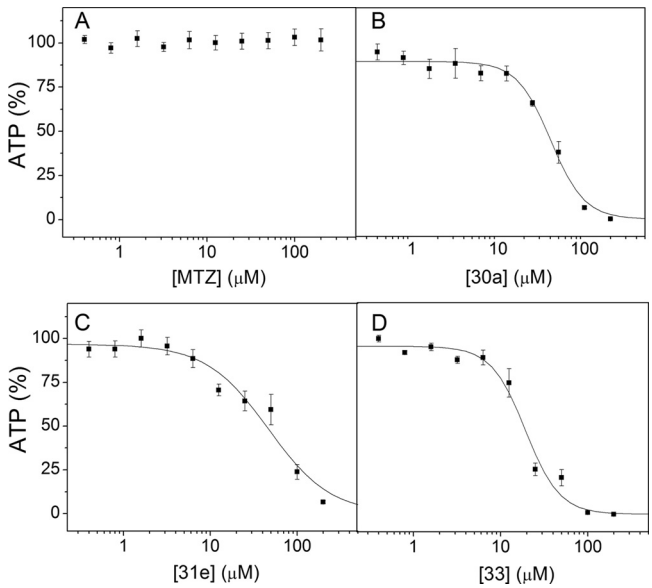

FIG 3 Dose-response curves of MTZ and the newly synthesized chalcone derivatives 30(a), 31(e), and 33 on Caco-2 cells. Data are expressed as means ± standard errors ($n \geq 12$).

ebbs and flows over time with every meal in order to meet metabolic demand. It is thus not surprising that the measured values for [O_2] in the different intestinal compartments are rather heterogeneous (see reference 14 and references therein). Despite this variability, however, relevant to this study, the proximal small intestine is a fairly oxygenated environment compared to other, more distal tracts of the gut (such as the sigmoid colon) (44–46), particularly at the epithelium level. O_2 concentration indeed decreases along a steep gradient moving inward from the intestinal submucosa, where up to 80 to 100 mm Hg O_2 has been reported, toward the nearly O_2-free luminal midpoint (14).

When testing new potential drugs against giardiasis, it is thus important to take O_2 into account, although this aspect has been almost completely neglected in the literature as yet. O_2 may indeed lead to changes in the metabolism of *Giardia* trophozoites, making the parasite more (or less) susceptible to specific drugs, and it may also react with and/or change the mechanism of action of a drug, thereby enhancing (or reducing) its antiparasitic effect. Moreover, O_2 is known to negatively affect the potency of MTZ, presumably by reacting with the redox-activated nitroradical form of this drug commonly used in the treatment of giardiasis (12). Testing the effect of O_2 on the activity of potential antigiardial drugs is therefore relevant also in the perspective of finding new antiparasitic compounds that are active under conditions in which MTZ potency is suboptimal.

In this study, 46 piperidine- and piperazine-based chalcones were synthesized with the aid of microwave irradiation at high purity and yield and tested for their *in vitro* antiparasitic activity against *G. intestinalis*. Drug susceptibility assays were performed according to reference 40, using ATP level as an indicator of trophozoite viability. The assay represents a valuable method for screening potential antigiardial compounds, as highlighted in recent studies (18, 20, 40). The synthesized compounds were initially tested in anaerobiosis so to allow a direct comparison with MTZ under conditions in which the latter drug is expected to kill *Giardia* trophozoites with maximal efficacy. Under these nonphysiological conditions, three compounds [30(a), 31(e), and 33], out of the 46 tested, were found to kill *Giardia* cells with IC_{50}s 2- to 3.5-fold greater than those of MTZ in the assay (Fig. 2). However, notably, when the assay was repeated with the three active compounds under more physiological microaerobic conditions, the presence of O_2 was shown to produce opposite effects on the new compounds compared to MTZ (Fig. 2). While the latter compound, as expected, showed a reduced toxicity toward parasitic cells, the three synthetic chalcone derivatives exhibited a 5- to 7-fold-higher antigiardial activity, compared to anaerobic conditions, and their IC_{50}s dropped well below (3- to 6-fold) the one measured for MTZ under the same microaerobic conditions (Table 2). Importantly, when comparing the antigiardial activities of the three active compounds [30(a), 31(e), and 33] under microaerobic conditions with their toxicity toward human Caco-2 cells (Fig. 3), compounds 30(a) and 31(e) proved to be much more active (25.3- and 15.6-fold, respectively) than toward human cells (Table 2). The antigiardial activity of compound 33 under the same microaerobic conditions was instead only 6.4-fold greater than its ability to kill human cells (Table 2). Cytotoxicity on human cells was evaluated at atmospheric O_2 level in order to ensure full O_2 activation of hit compounds.

Looking at the structure of these active compounds, the structural features accounting for the observed antigiardial activity cannot be easily inferred. The piperazine-based compounds 31(e) and 33 are active at variance from their piperidine-based analogues 30(e) and 32; thus, one may hypothesize that the piperazinic N in the 4-position may contribute to the observed activity. However, this does not seem to be the case, when comparing the active piperidine-based compound 30(a) with its inactive piperazine-based analogue 31(a). Furthermore, based on the information that any of the tested substitutions of the H atoms at $R_{1–5}$ in compound 30(a) leads to an activity loss (Table 1), it may be suggested that the steric hindrance at these substituents critically results in activity impairment. On the other hand, this conflicts with the observation that compound 31(e) with Cl at R_1 and R_5 exhibits high activity and its analogue 30(e) with H atoms at $R_{1–5}$ does not. Available data, therefore, do not allow us to unequivocally envisage the structural features underlying the antigiardial activity of the tested compounds, leaving open the possibility that the compounds found active in this study may have different molecular targets.

Despite these limitations, to the best of our knowledge, this is the first study in which new synthetic compounds have been comparatively assayed for their antigiardial activity, together with MTZ, under both anaerobic and microaerobic conditions, providing clear-cut evidence for an effect of O_2. The results show that the antiparasitic activity of a compound can be remarkably different in the presence of low, more physiological O_2 concentrations, compared to anaerobic conditions. This confirms that it is important to take O_2 into account when testing potential new drugs against *Giardia*.

At this stage, we can only speculate about the molecular mechanism that underlies the O_2-elicited antiparasitic activity of the novel synthetic chalcones tested in the present study. If the higher toxicity of these compounds observed in the presence of O_2 was resulting from the toxicity of O_2 itself directly damaging parasitic cells, *Giardia* trophozoites would have shown increased susceptibility to every compound tested in the assay, including MTZ, compared to anaerobic conditions. In contrast and at variance from the synthetic compounds, MTZ proved to be less effective in the presence of O_2 than under anaerobiosis, pointing to a specific effect on the hit synthetic compounds rather than to an unspecific toxicity of O_2 against *Giardia* cells. In this regard, it is intriguing that micromolar O_2 concentrations have been shown to produce profound changes in the metabolism of *Giardia* trophozoites, associated with a marked stimulation of both ethanol and CO_2 production, an oxidation of the intracellular NAD(P)H pool, and a strong inhibition of alanine production (47). Such a metabolic response to microaerobic growth conditions could be the basis of the higher susceptibility displayed by the parasite toward the synthetic compounds here investigated. An intriguing possibility, yet to be experimentally demonstrated, is that the active synthetic compounds described here specifically target a pathway(s) activated in the metabolic transition from anaerobic to microaerobic conditions.

In conclusion, by performing drug susceptibility assays both under anaerobic and more physiological microaerobic conditions, we identified two new compounds able to kill *Giardia* trophozoites in the presence of low O_2 concentrations selectively and more efficiently than MTZ. These two synthetic chalcone derivatives represent potential candidates for the design of novel antigiardial drugs. Importantly, the present study shows that the

presence or absence of O_2 is a crucial variable that should be taken into account when testing new potential drugs against *Giardia*.

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