Mechanism of trifluoromethionine resistance in Entamoeba histolytica

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Objectives: To determine the mechanism of trifluoromethionine resistance in Entamoeba histolytica and evaluate the impact of acquired drug resistance on virulence.

Methods: Trifluoromethionine-resistant amoebae were selected in vitro and examined for cross-resistance to antiamoebic drugs, stability of resistance, methionine γ-lyase (MGL) activity, cell adhesion and virulence. Targeted gene silencing was performed to confirm the role of EhMGL.

Results: Trophozoites with a resistance index of 154 were obtained. The cells were susceptible to chloroquine, metronidazole, paromomycin and tinidazole, but remained resistant to trifluoromethionine in the absence of drug pressure. A complete lack of EhMGL activity accompanied by increased adhesion and decreased cytolysis were also observed. Silencing of the EhMGL genes resulted in trifluoromethionine resistance.

Conclusions: This study provides the first demonstration of trifluoromethionine resistance in a parasitic protozoan. Repression of gene expression of drug targets represents a novel mechanism of resistance in E. histolytica. The information obtained from this work should help further development of trifluoromethionine derivatives that have lower chances of inducing resistance.

Keywords: amoebiasis, drug resistance, methionine γ-lyase

Introduction

Amoebiasis is an intestinal disease affecting 50 million people each year. Responsible for 110000 deaths annually, it occupies an important place on the list of parasitic causes of mortality worldwide. The aetiological agent is Entamoeba histolytica, and is contracted through the ingestion of cysts from food and water contaminated with faecal matter, or through annilingus. Inside a host, excystation occurs in the ileum, transforming cysts into trophozoites that may invade the colonic mucosal barrier. Invasive amoebiasis is generally treated with metronidazole, a unique drug that is effective against luminal and tissue trophozoites. Treatment failures, however, have been reported, and while the factors responsible are not clear, differences in drug susceptibility, resistance, virulence and host immune response have been implicated.

While there are no reports of high levels of resistance to metronidazole in clinical isolates, resistance to the drug has been demonstrated in vitro. In addition, resistance to the drug in other anaerobic and microaerophilic parasitic protozoa, such as Giardia lamblia and Trichomonas vaginalis, is well documented. These findings signal forthcoming metronidazole resistance in E. histolytica and have spurred the search for alternative chemotherapeutic agents.

Trifluoromethionine or L-S-(trifluoromethyl)homocysteine is a fluorinated derivative of L-methionine. Its antimicrobial activity was first reported by Zygmunt and Tavormina in 1966 when they observed that low trifluoromethionine concentrations completely inhibited microbial growth. Since then, trifluoromethionine has been shown to be effective against several pathogenic microorganisms, including E. histolytica, where the drug reportedly killed axenic cultures after 72 h. It has been assumed that in these organisms trifluoromethionine is degraded by a unique enzyme called methionine γ-lyase (MGL) into α-ketobutyrate, ammonia and trifluoromethanethiol (CF3SH). The last product is unstable under physiological conditions and breaks down to carbonothionic difluoride (CSF2), a reactive cross-linker of primary amine groups, which is toxic to cells.

Trifluoromethionine is currently being developed as an alternative chemotherapeutic agent against E. histolytica, but
its potential to induce resistance has not yet been demonstrated.\textsuperscript{14} Thus, the aim of this work is to determine whether \textit{E. histolytica} resistant to the drug can be selected in vitro and investigate its underlying mechanism.

**Materials and methods**

**Chemicals and drugs**

Production of trifluoromethionine was previously described.\textsuperscript{14} Metronidazole, chloroquine, paromomycin, L-methionine, L-cysteine, trichloroacetic acid (TCA) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) were purchased from Sigma-Aldrich (St Louis, MO, USA), while Opti-MEM medium, TRIzol reagent, SuperScript III First-Strand Synthesis System, PLUS reagent, Lipofectamine and geneticin (G418) were acquired from Invitrogen (Carlsbad, CA, USA), Tinidazole and pyridoxal 5′-phosphate (PLP) were acquired from LKT Laboratories, Inc. (St Paul, MN, USA) and Nakarai Chemicals, Ltd (Kyoto, Japan), respectively. All other chemicals were obtained from Wako Pure Chemical (Osaka, Japan) unless otherwise stated.

**Parasites and cultivation**

\textit{E. histolytica} strain HM-1:IMSS cl6 (HM-1) and G3 strain, kindly given by David Mirelman (Weisman Institute, Israel), were cultured axenically in BI-S-33 medium for 48–72 h at 35.5°C in 13×100 mm Pyrex screw cap culture tubes or 25 cm\textsuperscript{2} tissue culture flasks (#152904; Nunc, Roskilde, Denmark).\textsuperscript{16}

**Cultivation and generation of a trifluoromethionine-resistant \textit{E. histolytica} line**

Semi-confluent cultures of the HM-1 strain were exposed to 0.1 mg/L (0.5 \(\mu\)M) trifluoromethionine for 24 h. Spent medium and dead cells were removed by aspiration and replaced with fresh medium without the drug and cultured until the mid-logarithmic phase. The procedure was repeated, followed by a stepwise (0.2, 0.5 or 1 mg/L) increase in drug concentration until cells growing at 4 mg/L (20 \(\mu\)M) trifluoromethionine were obtained, designated as the trifluoromethionine-resistant (TFMR) strain.

**Cultivation of Chinese hamster ovary (CHO) cells**

CHO cells were grown in Ham’s F-12 medium (GIBCO, Invitrogen Co., Auckland, New Zealand) supplemented with 10% fetal calf serum (Medical Biological Laboratory International, Woburn, MA, USA) in 25 cm\textsuperscript{2} canted-neck culture flasks (IWAKI, Tokyo, Japan) at 37°C with humidified air and 5% CO\textsubscript{2}. For monolayer experiments, CHO cells were grown to confluency either in 24-well plates (Costar Co., Cambridge, MA, USA) or 35 mm glass-bottom culture dishes coated with collagen (MatTek Co., Ashland, MA, USA).

**Growth kinetics of the TFMR strain and half maximal inhibitory concentration (IC\textsubscript{50}) of trifluoromethionine and unrelated drugs**

Approximately 1×10\textsuperscript{5} cells/mL trophozoites were inoculated in 6 mL of culture medium. Cultures used for growth kinetics were maintained with or without 4 mg/L trifluoromethionine and examined every 24 h for 120 h. The number of viable cells was counted in duplicate cultures by Trypan Blue exclusion assay.\textsuperscript{17} Briefly, cultures were incubated on ice for 10 min and 50 \(\mu\)L of cell suspension was transferred to a 1.5 mL tube and centrifuged at 100 g for 5 min at 4°C. The pellet was resuspended in 50 \(\mu\)L of PBS, pH 7.4, containing 2 mg/mL Trypan Blue. The mixture was incubated at room temperature for 3 min and 25 \(\mu\)L was applied to a haemocytometer and counted.

Cultures used to determine IC\textsubscript{50} were treated separately with 15 concentrations of trifluoromethionine, chloroquine, metronidazole, paromomycin and tinidazole, with a 2-fold increase between doses ranging from 0.04 mg/L to >400 mg/L. After 48 h of incubation, spent medium was aspirated and replaced with 6 mL PBS. Tubes were placed on ice for 10 min and 25 \(\mu\)L was applied to a haemocytometer and counted. The IC\textsubscript{50} was determined by fitting a non-linear regression curve to the concentration–percentage survival growth curve. All experiments were repeated three times with two replicates per experiment.

**Measurement of \textit{E. histolytica} adhesion**

Trophozoites in the exponential growth phase were harvested, washed with cold PBS and centrifuged at 500 g for 5 min at 4°C. CHO cells were trypsinized (10 mg/mL EDTA, pH 7.4, containing 5 mg/mL trypsin) in F-12 medium for 10 min, harvested by centrifugation at 500 g for 5 min at 4°C and washed with PBS. Amoebae and CHO cells were resuspended in Opti-MEM medium supplemented with 5 mg/mL L-cysteine and 1 mg/mL ascorbic acid. Amoebae (1×10\textsuperscript{5} cells) and CHO cells (2×10\textsuperscript{3} cells) were mixed in 1 mL of medium, centrifuged at 500 g for 5 min at 4°C and incubated on ice for up to 90 min. Following incubation, 0.8 mL of supernatant was removed and the pellet broken up by repeated rotation of the tube by hand. A drop of the cell suspension was examined as described above. All the trophozoites were examined and those with at least three CHO cells attached were considered adherent.\textsuperscript{18} A plate adhesion assay was performed as previously described.\textsuperscript{19} Briefly, ~1×10\textsuperscript{5} trophozoites were seeded into a well of a 96-well plate coated with either human fibronectin (BD BioCoat Cell Environment; BD Biosciences, San Jose, CA, USA) or collagen type I (SigmaScreen; Sigma-Aldrich) and incubated under anaerobic conditions using Anaero-Cult A (Merck, Darmstadt, Germany) for up to 40 min at 35.5°C. The medium was removed and non-adherent cells were gently washed twice with PBS warmed to 35.5°C. Adherent cells were fixed with 40 mg/mL paraformaldehyde (TAAB Laboratories, Aldermaston, England) for 10 min and washed twice with PBS. Cells were stained with 1 mg/mL methylene blue in 100 mM borate buffer, pH 8.7, for 20 min and washed twice with distilled water. The stain was extracted with 200 \(\mu\)L of 20 mg/mL SDS and the absorbance measured at 660 nm using a DU 530 Spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

**CHO monolayer destruction assay**

CHO monolayer destruction was measured as described previously with minor modifications.\textsuperscript{20} Briefly, 1×10\textsuperscript{6} CHO cells were seeded into 24-well plates and grown to confluency in a humidified incubator at 37°C and 5% CO\textsubscript{2}. The medium was removed and the plates were washed with supplemented Opti-MEM medium. Approximately 2×10\textsuperscript{5} cells of the TFMR and wild-type strains were resuspended in 2 mL supplemented Opti-MEM medium and added to each well. The plates were incubated under anaerobic conditions at 35.5°C for up to 120 min. The plates were placed on ice for 10 min to release adhered trophozoites and washed twice with cold PBS. The number of CHO cells remaining in the wells was measured as described above in the \textit{E. histolytica} plate adhesion assay.

**Substrate gel electrophoresis**

Proteinase activity was detected by substrate gel electrophoresis, as described previously.\textsuperscript{21} Briefly, 20 \(\mu\)g of cell lysates from the TFMR and wild-type strains were separated in a 12% (w/v) SDS–polyacrylamide
gel copolymerized with 0.1% (w/v) gelatin. The gel was incubated in 2.5% (v/v) Triton X-100 for 1 h and then in 100 mM sodium acetate, pH 4.5, 1% (v/v) Triton X-100 and 20 mM dithiothreitol (DTT) for 3 h at 37°C. The bands were visualized after staining with 0.5% (w/v) Coomassie Brilliant Blue R-250.

**EhMGL activity assay**

EhMGL activity was measured based on the production of α-ketobutyrate, as previously described. Briefly, ~2 × 10⁷ trophozoites of the wild-type and TFMR strains were suspended in 500 µL of 100 mM sodium phosphate buffer, pH 7.0, 20 mM PLP, 0.5 mM E-64, 0.1 mM phenylmethylsulfonyl fluoride and Complete Mini-EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The suspension was subjected to three freeze–thaw cycles and insoluble materials were separated by centrifugation at 15,000 g for 15 min at 4°C. Protein concentration was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), with BSA as standard. The reaction mixture contained 100 mM sodium phosphate buffer, pH 7.0, 20 µL PLP, 1 mM DTT, 4 mM trifluoromethionine and 1 µg/mL amoeba lysate. The reaction was initiated by adding trifluromethionine and terminated, after 20 min incubation at 37°C, by adding 500 mg/mL TCA. The mixture was centrifuged at 15,000 g for 10 min at 4°C and 100 µL of supernatant was incubated with 160 µL of 333 mM sodium acetate, pH 5.0 and 1.5 mM of MBTH for 60 min at 50°C. The amount of α-ketobutyrate generated was determined by measuring the absorbance at 320 nm with sodium 2-oxobutyrate (Fluka BioChemika, Buchs, Switzerland) as standard.

**Immunoblot analysis**

Cell lysates were prepared as described above, with all subsequent incubations performed at room temperature. Total protein (10–30 µg) was separated on a 12% (w/v) SDS–polyacrylamide gel and subsequently electrotransferred onto nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences, Little Chalfont, Bucks, UK), as described previously. Membranes were blocked Incubation with 160 µL of 333 mM sodium acetate, pH 5.0 and 1.5 mM of MBTH for 60 min at 50°C. The amount of α-ketobutyrate generated was determined by measuring the absorbance at 320 nm with sodium 2-oxobutyrate (Fluka BioChemika, Buchs, Switzerland) as standard.

**Quantitative real-time (qRT)–PCR**

Semi-confluent cultures of the wild-type and TFMR strains maintained with or without 4 mg/L trifluoromethionine were harvested as described above. Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions. RNA quality was assessed with the Experion automated electrophoresis system using the Experion RNA StdSens analysis kit (Bio-Rad). RNA quantity was determined by measuring absorbance at 260 nm with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The synthesis of cDNA was performed using the SuperScript III First-Strand Synthesis System according to the manufacturer’s instructions. The cDNA synthesis was completed on a DNA Engine Peltier Thermal Cycle (Bio-Rad).

The Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used for qRT–PCR in accordance with the manufacturer’s instructions. In addition to the EhMGL1 and EhMGL2 primers used in this study, a housekeeping gene, the RNA polymerase II gene (EhRNA-PII), was used as a control (Table 1). Each PCR contained 5 µL of cDNA and 15 µL primer mix, composed of 10 µL of 2X Fast SYBR Green Master Mix, sense and antisense primers and nuclease-free water, to bring the volume to 20 µL. qRT–PCR was performed using StepOne Plus Real-Time PCR System (Applied Biosystems) with the following cycling conditions: enzyme activation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s. All test samples were run in triplicate, including an RT-negative control for each sample set and a blank control consisting of nuclease-free water in place of cDNA. Quantification for each target gene was determined by the ΔΔCt method with EhRNA-PII as the reference gene.

**Table 1.** Primers used to quantify EhMGL1 and EhMGL2 by qRT–PCR and construct the plasmids for targeted gene silencing of EhMGL1 and EhMGL2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’–3’)</th>
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</thead>
<tbody>
<tr>
<td>qRT–PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EhMGL1</strong></td>
<td>sense</td>
<td>GTACTTTAGCAAGTTTCTACCTTGAGTG</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>CCAACGGGAAAATTCTAATTTACTGAGG</td>
</tr>
<tr>
<td><strong>EhMGL2</strong></td>
<td>sense</td>
<td>TCCGCCAGGTCTAAATTTGCTATG</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>AAGTTCGAAGAGAAACAGCTATATCC</td>
</tr>
<tr>
<td><strong>EhRNA-PII</strong></td>
<td>sense</td>
<td>GATCACAATATCTCTAAACACA</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>TCAATTATTTTTGACCGTCGCTTCC</td>
</tr>
<tr>
<td>Gene silencing</td>
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<td><strong>EhAP-A</strong></td>
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<td>AGCTCTAGACCCGCGCGCCGCTTCGACCTTTTG</td>
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<td></td>
<td>antisense</td>
<td>CTCGAGCTCGTTAAAGGCGCCTAGTGGTGTAAGATAG</td>
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<tr>
<td><strong>EhMGL1</strong></td>
<td>sense</td>
<td>AGCTAGGCGCCATTGAGCTGAAAGATTATGAGTC</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>GCCATCGGCTCCTAAAAGGAGGATTTAC</td>
</tr>
<tr>
<td><strong>EhMGL2</strong></td>
<td>sense</td>
<td>AGCTAGGCGCTATGTCCATTGGAAGGATTTAC</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>GCCATCGGCTCACCATATCTTTAATCTATTTCC</td>
</tr>
</tbody>
</table>

Restriction sites for SacII are marked by single underlining, restriction sites for SacI are marked by double underlining, and restriction sites for StuI are marked by broken underlining.
Production of EhMGL gene-silenced strains

The vector used for targeted gene silencing was constructed by amplifying the 5′ upstream region of the EhAP-A gene from psAP-2, using sense and antisense EhAP-A primers containing restriction sites for SacI, SacII and StuI at the 5′ end (Table 1). The PCR product was ligated into psAP-2 and the resulting plasmid was named psAP-2-Gunma vector. The EhMGL1 and EhMGL2 genes were amplified by PCR from cDNA derived from the wild-type strain using specific primers (Table 1). The PCR products and psAP-2-Gunma vector were digested with StuI and SacI, and ligated to generate the EhMGL1 and EhMGL2 gene-silencing plasmid. These plasmids were introduced separately into trophozoites of the G3 strain by lipofection, with minor modifications as previously described.24 Briefly, 5×10⁵ cells suspended in 5 mL supplemented Opti-MEM medium were seeded into a 12-well plate and incubated under anaerobic conditions at 35.5°C for 30 min. Following incubation, 4.5 mL of medium from each well was removed and 500 μL of liposome/plasmid mixture (5 μg of plasmid, 10 μL of PLUS reagent and 20 μL of Lipofectamine in Opti-MEM medium) was added. After 5 h of transfection, cells were harvested by placing the plate on ice for 15 min, then they were added to culture tubes with 5.5 mL of cold BI-S-33 medium and incubated at 35.5°C for 24 h. Transformants were selected by adding 2 μg/mL geneticin to the cultures and gradually increasing the drug concentration to 6 μg/mL.

Statistical analysis

Correlation coefficients were calculated using the Student’s t-test function of the Microsoft Excel statistical package (Microsoft Corp., Redmond, WA, USA). Probability levels (P) <0.05 were considered significant. IC50 was calculated by non-linear regression analysis using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Establishment of the TFMR strain

Exposure of wild-type trophozoites of the HM-1 strain to drug concentrations >1 mg/L (5 μM) caused changes in cell morphology, e.g. rounding and detachment within 24 h, and eventually resulted in death after 72 h. However, when we initiated the culture with permissive concentrations, e.g. 0.1 mg/L (0.5 μM) of trifluoromethionine, and gradually increased the drug concentration over 6 months, the TFMR strain, which could be maintained at 4 mg/L (20 μM) with growth rates similar to those of the parental susceptible strain, was obtained.

Growth kinetics and IC50 of the TFMR strain

The TFMR strain showed comparable growth kinetics with or without 4 mg/L trifluoromethionine (Figure 1a). Its population doubling time was slightly longer than the wild-type when cultured without the drug (13.3±1.1 h and 9.6±1.4 h, respectively). As reported previously, 4 mg/L trifluoromethionine caused significant growth inhibition in the wild-type as early as 24 h and a cytolytic effect within 72 h.15,16 The growth rate of the TFMR strain in the presence of the drug, 14.2±1.5 h, was not significantly different from those cultured without the drug (P>0.05).

A significantly different drug concentration versus percentage survival profile was observed between resistant and susceptible cells (Figure 1b). The IC50 of trifluoromethionine in the TFMR and parental strain was 197.4±20.9 and 1.3±0.2 mg/L, respectively, and its resistance index was 154 (Table 2).

Trifluoromethionine resistance is irreversible upon the removal of the drug

We further maintained the TFMR strain in the presence or absence of 4 mg/L trifluoromethionine. Growth inhibition by trifluoromethionine of TFMR and wild-type strains. Values shown are the means (±SEM) of at least two replicate determinations from three independent experiments. TFM, trifluoromethionine.

and parental strain was 197.4±20.9 and 1.3±0.2 mg/L, respectively, and its resistance index was 154 (Table 2).

Cross-resistance

The IC50 and resistance indices of unrelated drugs were computed to determine whether the TFMR strain exhibited cross-resistance (Table 2). Results showed that the TFMR strain had...
susceptibilities comparable to wild-type towards chloroquine, metronidazole, paromomycin and tinidazole. These observations indicated that the mechanisms of action and resistance of trifluoromethionine are different for these drugs.

Trifluoromethionine resistance affects cell adhesion and virulence

While 54.8 ± 4.1% of TFMR cells adhered to three or more CHO cells, 42.2 ± 3.6% of wild-type cells attached to three or more CHO cells (P < 0.05; Figure 2a). The TFMR strain also showed 43.1 ± 19.2% (P < 0.01) or 23.5 ± 2.8% (P < 0.05) better attachment to fibronectin- or collagen-coated wells compared with the wild-type strain, respectively (Figure 2a). The TFMR strain, however, destroyed CHO monolayers at a slower rate, particularly at early timepoints (P < 0.05 at 30 min; Figure 2b), indicating a slight reduction in cytopathy. This was consistent with the observed decrease in intensity of the band corresponding to EhCP5, a well-established virulence determinant, in the zymogram (Figure 2c). No gelatin degradation was observed when lysates were treated with E-64 (data not shown).

Repression of EhMGL in the TFMR strain

EhMGL activity was not detected in the TFMR strain (Figure 3a). Repression of the enzyme was verified by western blot with anti-EhMGL1 and anti-EhMGL2 antibodies (Figure 3b). Analysis of mRNA steady-state level by qRT–PCR also indicated that transcription of both EhMGL1 and EhMGL2 were repressed (Figure 3c).

Repression of EhMGL is sufficient for trifluoromethionine resistance

These observations suggested that repression of EhMGL activity alone could result in drug resistance. To answer this question, we created EhMGL1 and EhMGL2 gene-silenced strains. The expression of both EhMGL1 and EhMGL2 were abolished in the gene-silenced strains (Figure 3d). The IC50 value of trifluoromethionine and the resistance index of the EhMGL1 and

Table 2. IC50 of trifluoromethionine and drugs commonly used to treat amoebiasis against TFMR

<table>
<thead>
<tr>
<th>Drug</th>
<th>PubChem CID no.</th>
<th>IC50 (mg/L)</th>
<th>Resistance indexa</th>
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<tbody>
<tr>
<td>TFMR</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>chloroquine</td>
<td>64927</td>
<td>8.18 ± 4.08</td>
<td>0.4</td>
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<tr>
<td>metronidazole</td>
<td>4173</td>
<td>0.34 ± 0.29</td>
<td>13.1</td>
</tr>
<tr>
<td>paromomycin</td>
<td>24176</td>
<td>10.70 ± 2.96</td>
<td>3.1</td>
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<tr>
<td>tinidazole</td>
<td>5479</td>
<td>0.67 ± 0.30</td>
<td>1.1</td>
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<tr>
<td>trifluoromethionine</td>
<td>165196</td>
<td>197.43 ± 20.90</td>
<td>154.0</td>
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<tr>
<td>MGL1gs</td>
<td></td>
<td></td>
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<tr>
<td>trifluoromethionine</td>
<td>165196</td>
<td>670.09 ± 41.78</td>
<td>198.7</td>
</tr>
<tr>
<td>MGL2gs</td>
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<td>trifluoromethionine</td>
<td>165196</td>
<td>559.61 ± 92.04</td>
<td>165.9</td>
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</table>

*Resistance index values are determined by dividing the IC50 of drug in TFMR by that in HM-1. No cross-resistance was observed.

bMGL1gs and MGL2gs refer to trophozoites transfected with EhMGL1 and EhMGL2 gene-silencing plasmids.

Figure 2. Effect of trifluoromethionine resistance on adhesion, cytolysis and cysteine protease secretion. (a) Effect of trifluoromethionine resistance on adhesion to CHO cells, fibronectin-coated plates or collagen-coated plates. Three assays were used to compare the adhesive capacities of the TFMR and wild-type strains. Microscopic examination of trophozoite attachment to CHO cells and colorimetric determination of cells on coated plates both show that the TFMR strain adheres more strongly compared with the parental strain. (b) Effect of trifluoromethionine resistance on cytolysis of CHO cells. The TFMR strain destroyed CHO monolayers at a slower rate, particularly at early timepoints. All experiments were repeated three times with two replicates per experiment. (c) Effect of trifluoromethionine resistance on cysteine protease secretion. Arrowhead indicates EhCP5.
amoebic liver abscess in a rodent model. While these oral administration of the drug prevented the formation of 2050 choice to treat amoebiasis. Recently, however, it has become For more than 45 years, metronidazole has been the drug of Discussion TFMR strain (Table 2).

EhMGL2 gene-silenced strains were comparable to that of the TFMR strain (Table 2).

**Figure 3.** Repression of EhMGL expression in TFMR and EhMGL gene-silenced strains. (a) Repression of EhMGL activity in the TFMR strain. EhMGL activity was measured based on the production of α-ketobutyrate (nmol/min/mg). Reactions were carried out with either 1 μg/mL lysate of the TFMR or wild-type strain and 4 mM trifluoromethionine. EhMGL activity was not detected (ND) in TFMR (P < 0.01). (b) Repression of EhMGL in TFMR cultured in the presence or absence of trifluoromethionine. (c) EhMGL mRNA expression ratios of the TFMR strain relative to HM-1. Quantification of each target gene was determined by the ΔΔCt method using the housekeeping gene Ehrnapii as a control. (d) Repression of EhMGL protein in EhMGL gene-silenced strains was detected using methods similar to (b). All experiments were performed at least three times. TFM, trifluoromethionine.

**Discussion**

For more than 45 years, metronidazole has been the drug of choice to treat amoebiasis. Recently, however, it has become clear that *E. histolytica* is capable of developing resistance to the drug. We previously demonstrated the potential of trifluoromethionine as an alternative chemotherapeutic agent against amoebiasis. We showed that trifluoromethionine effectively killed trophozoites in vitro and that a single subcutaneous or oral administration of the drug prevented the formation of amoebic liver abscess in a rodent model. While these results demonstrate the effectiveness of the drug, its clinical application may be complicated by the parasite’s capacity to develop drug resistance, which has not yet been investigated.

In principle, the evolutionary pathways of drug resistance can be reconstructed in vitro and, in this work, we showed that exposure of trophozoites to low levels of trifluoromethionine, followed by a gradual stepwise increase in drug concentration, leads to selection of the TFMR strain. In view of this finding, our research was directed towards the characterization of this cell line and identification of factors responsible for resistance. As shown in Figure 1(a), there was a slight delay in the growth rate of the TFMR strain compared with wild-type cells in the absence of the drug. When resistance is caused by alteration of a target, metabolic costs are expected, and this fitness loss is often reflected in reduced growth. We next examined if the phenotype could be reversed in the absence of the drug, which is the strategy likely to be favoured by selection to avoid a permanent cost to fitness. The TFMR strain, however, remained resistant to the drug after more than a year of culture in drug-free medium (data not shown). This often occurs when the target is not essential for survival or if compensatory mechanisms exist.

Some studies have shown that cell lines resistant to one drug are often cross-resistant to closely related drugs or to unrelated compounds when they carry membrane alterations or when their mode of action is the same. It was therefore not surprising to find that the TFMR strain was not cross-resistant to the drugs listed in Table 2. Trifluoromethionine is a fluorinated methionine analogue and its mode of action is clearly unique to microaerophilic anaerobic microorganisms that possess MGL.

In *E. histolytica*, differential adhesive capacity and virulence are important factors that determine the establishment and outcome of infection. Studies have shown that these factors are influenced by drug resistance, and vice versa. It is conceivably that the increased adhesion observed in the TFMR strain was necessary in selecting cells during the initial stages of drug treatment. Adhesion of cells together may create a specialized microenvironment that confers a significant advantage that promotes survival when challenged by trifluoromethionine, and could be the first step in the pathway towards resistance. Fitness loss due to acquired resistance is also reflected in decreased invasiveness. The TFMR strain had slower CHO monolayer destruction, and while the reason for this phenotype is not entirely clear, it may be at least in part attributable to a decrease in the activity of cysteine proteases, particularly *EhCP5*, which has been implicated in the parasite’s cytotoxic effects on mammalian cells. As shown in Figure 2(c), the TFMR strain had lower *EhCP5* levels compared with the wild-type. Since we also determined that its mRNA level was not differentially modulated (data not shown), this indicates a possible defect in enzyme processing or trafficking. Currently it is not known if the factors that determine resistance and adherence/cytodysplasia of the strain are genetically linked.
Previously it was shown that the amoebicidal effect of trifluoromethionine ceases when trophozoites are co-incubated with dl-propargylglycine (PPG), an inhibitor of EhMGL. This finding indicates that the enzyme is part of the cytotoxic mechanism of the drug and also suggests that its suppression or inactivation is a key factor in drug resistance, as shown by the phenotype of EhMGL gene-silenced strains toward the drug (Table 2). It is not known how this repression took place in the TFMR strain during drug treatment. However, clues were obtained from our observation that resistance was irreversible in the absence of the drug, and that this was associated with the continued repression of EhMGL (Figure 3b and c). It is likely that EhMGL was repressed at the transcriptional level and this repression could have involved epigenetic silencing. This mechanism was suggested by the high frequency of trophozoites that survived in low trifluoromethionine concentrations (data not shown). It is known that most drug-induced epigenetic repression is inherited from one generation to the next and that DNA hypermethylation constitutes one response of cells to drugs. In E. histolytica, transcriptional gene silencing may also result from histone modifications that create an environment of heterochromatin around a gene that makes it inaccessible to the transcriptional machinery. It is also possible that trifluoromethionine or one of its degradation products may have interfered with the binding of transcription factors to their respective cognate cis element, preventing the transcription of EhMGL genes. Epigenetic gene silencing was also suggested when we failed to restore trifluoromethionine susceptibility in the TFMR strain by episomal expression of EhMGL. It was previously reported in E. histolytica that transcriptional silencing of the gene coding for amoebapore A resulted in the silencing of both intrinsic chromosomal and ectopically introduced episomal genes. We also attempted to restore EhMGL activity by the treatment of TFMR cultures with 5-azacytidine, an inhibitor of DNA methylation, but observed the continued repression of EhMGL. The last observation implied that DNA methylation may not be involved in the silencing of EhMGL, but the possible involvement of other types of methylation remains unknown. Whether DNA hypermethylation or histone modification is responsible for EhMGL repression in the TFMR strain requires further investigation, but what is clear is the non-essential role of EhMGL activity for viability in vitro. It is possible that its absence was tolerated because of a compensatory mechanism occurring elsewhere in the genome that remains to be identified.

Suppression of enzyme activity is one mechanism that leads to drug resistance, especially if cells are not severely penalized by losing its function. Initially there could have been an adaptive conflict between the development of resistance and the maintenance of sufficient EhMGL activity. However, because of the specificity of trifluoromethionine to EhMGL, selection took one course that resulted in complete silencing of the gene. In this study we have provided evidence that in E. histolytica, EhMGL repression is the key factor that leads to resistance to the drug.

The potential of trifluoromethionine as an amoebicide, however, remains undeniable. Its chemotherapeutic index is high and its activity is not limited to E. histolytica, as it is active against all microaerophilic/anaerobic organisms that possess MGL. While we showed that resistance to the drug can be induced in vitro, the same may not necessarily be true in vivo, where both drug and host factors come into play. The results presented here may provide clues on how to redesign trifluoromethionine so it is less likely to promote resistance and to help preserve its efficacy by developing appropriate therapeutic protocols.

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Transparency declarations
None to declare.

References
domains in the parasitic protozoon via a unique modular protein consisting of RhoGEF/DH and FYVE phosphates mediate cytoskeletal reorganization during phagocytosis.

