International Journal of Antimicrobial Agents 35 (2010) 56–61

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Cytotoxic effect of amide derivatives of trifluoromethionine against the enteric protozoan parasite *Entamoeba histolytica*

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ARTICLE INFO

Article history:
Received 23 April 2009
Accepted 3 August 2009

Keywords:
Drug discovery
Sulphur-containing amino acid metabolism
Methionine γ-lyase
Protozoan parasite

ABSTRACT

Amoebiasis, caused by infection with the enteric protist *Entamoeba histolytica*, is one of the major parasitic diseases. Although metronidazole and its derivatives are currently employed in therapy, the paucity of effective drugs and potential clinical resistance necessitate the development of a novel drug. Trifluoromethionine (TFM) is a promising lead compound for antiamoebic drugs. To potentiate the antiamoebic effect of TFM, we synthesised various amide derivatives of TFM and evaluated their cytotoxicity. The amide derivatives of TFM were observed to have a superior cytotoxic effect compared with TFM and metronidazole against *E. histolytica* in vitro. Although TFM showed cytotoxicity following degradation by methionine γ-lyase, the derivatives were degraded by the enzyme less efficiently compared with TFM. We further demonstrated that a representative derivative was hydrolysed by the amoebic cell lysate to first yield TFM, followed by degradation similar to TFM. Hydrolysis was partially inhibited by protease inhibitors. A single subcutaneous or oral administration of TFM and its amide derivatives also effectively prevented the formation of amoebic liver abscess in a rodent model. These data demonstrate the improved effectiveness of TFM derivatives against *E. histolytica* infection and elucidate the mechanisms underlining the mode of action of these compounds.

1. Introduction

Amoebiasis is an infectious disease caused by the enteric protozoan parasite *Entamoeba histolytica* and is the second leading cause of death from parasitic diseases after malaria. Only metronidazole and related compounds are commonly used against invasive intestinal and extraintestinal amoebiasis [1,2]. Although clinical resistance against metronidazole has not yet been demonstrated, sporadic cases of treatment failure have been reported [1]. In addition, it has been shown that this parasite easily adapts to therapeutic levels of metronidazole in vitro [3]. Resistance to metronidazole is also acquired easily by many bacterial species as well as *Giardia intestinalis* and *Trichomonas vaginalis* [1]. Therefore, the development of a novel antiamoebic drug is urgently required.

Pathways present exclusively in microorganisms but missing in humans may potentially represent a rational target for drug development. *Entamoeba histolytica* has several unique metabolic features. It lacks both the forward and reverse trans-sulphuration pathways that convert methionine to cysteine via cystathionine or vice versa, whilst it possesses L-methionine γ-lyase (MGL) (EC. 4.4.1.11) to decompose methionine, homocysteine and cysteine and produces ammonia, α-keto acid and volatile thiols [4]. MGL is present in only limited lineages of bacteria, parasitic protozoa and plants but is absent in mammals (see in [5]). In *E. histolytica*, two isoenzymes of MGL (MGL1 and MGL2) with distinct substrate specificities have been identified [5].

Trifluoromethionine (TFM), also know as *S*-trifluoromethyl-L-homocysteine, a fluorinated analogue of methionine, has been

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doi:10.1016/j.ijantimicag.2009.08.016

shown to be highly toxic to bacteria, *Porphyromonas gingivalis*, *T. vaginalis* and *E. histolytica* in vitro [6–9]. It was also reported to treat infections by *P. gingivalis* and *T. vaginalis* in a rodent model [7,8]. In the present study, the in vitro and in vivo efficacy of TFM and its derivatives were examined to gain insight into the structure–activity relationship and to determine the mechanism underlying their mode of action.

2. Materials and methods

2.1. Chemical synthesis of trifluoromethionine and its derivatives

The synthetic scheme and structure of TFM and its analogues are shown in Supplementary Fig. 1.

2.2. Parasites and cultivation

Trophozoites of *E. histolytica* HM-1:IMSS cl6 were cultured axenically in BIS medium at 35.5 °C as described previously [10].

2.3. In vitro cytotoxicity assay of trifluoromethionine derivatives against Entamoeba histolytica trophozoites and Chinese hamster ovary (CHO) cells

Approximately 5×10^3 amoebae in 280 μL of medium were seeded into each well of a 96-well plate and incubated with various concentrations of TFM derivatives for 48 h. Following incubation, the amoebae were washed with 100 μL of pre-warmed Opti-MEM® I (Invitrogen, Carlsbad, CA) and viable cells were counted using Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany). Cytotoxicity against mammalian cells was examined as described above except that 1×10^4 CHO cells were cultivated in 280 μL of F-12 medium (Invitrogen) under 5% CO_2 at 37 °C.

2.4. Assay for L-methionine γ-lyase activity

Recombinant MGL1 (3 μg) or MGL2 (1.2 μg) (final concentrations 15 μg/mL and 6 μg/mL, respectively) [5] was incubated with 1 mM TFM, anilide (TFM-01) or benzylamide (TFM-02) in 200 μL of 100 mM sodium phosphate buffer (pH 7.2) containing 20 μM pyridoxal-5′-phosphate (PLP) and 2.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma, St Louis, MO) at 37 °C. Released thiols were measured as described previously [9].

2.5. Degradation of trifluoromethionine derivatives in amoebic cell lysate determined by capillary electrophoresis with electrospray ionisation time-of-flight mass spectrometry (CE-TOFMS)

Approximately 3×10^6 trophozoites were lysed with 450 μL of 10 mM HEPES (pH 7.5) containing 1 μM PLP and 0.05% Tween 20. The filtrated supernatant (50 μg) was incubated with 2 mM TFM-01, OB-01 (see Supplementary Fig. 1) or dimethyl sulphoxide (DMSO) at 37 °C for 2 h. The reaction was terminated by adding 10 times volume of methanol. TFM and 2-oxobutyrate were quantified by CE-TOFMS [11] in cationic and anionic mode, respectively, under conditions described previously [11]. TFM and sodium 2-oxobutyrate were used as standards.

2.6. Evaluation of the amoebicidal activity of trifluoromethionine derivatives using a hamster liver abscess model

Approximately 1×10^6 trophozoites were injected into the left lobe of the liver of 2–3-week-old Syrian hamsters (mean ± standard error body weight, 39.9 ± 0.56 g). TFM or its derivatives dissolved in DMSO were administered either subcutaneously (0.1 mL, 0.2 μmol/animal) or orally through a stomach catheter (0.5 mL, 0.2–1.0 μmol/animal) at 24 h after infection. Animals were sacrificed 6 days post infection and the liver and abscesses were dissected and weighed separately.

3. Results and discussion

The in vitro cytotoxicities of TFM, 15 TFM derivatives and 4 control compounds lacking fluorine were evaluated by measuring their 50% inhibitory concentration (IC_{50}) values against *E. histolytica* trophozoites (Table 1). Whilst TFM showed an IC_{50} of 7.34 μM, the derivatives TFM-01 and TFM-02 had IC_{50} values of 2.22–2.28 μM. Three difluoroanilide compounds [2,3-difluoroanilide (TFM-03), 2,6-difluoroanilide (TFM-04) and 2,5-difluoroanilide (TFM-05)] also had a comparable 2.5–3.0-fold reduction in their IC_{50} values compared with TFM. Three derivatives with 2,5- and 3,4-dimethoxyanilide (TFM-07 and TFM-08) or 3,4,5-trimethoxyanilide (TFM-09) modification had a further 2-fold improvement in their IC_{50} values. These values are 1.6–4.3-fold lower than metronidazole (mean ± standard deviation in triplicate, 4.76 μM ± 0.22). In contrast, compounds containing additional functional groups on the phenyl ring of TFM-01 [2-methyl-4-chloroanilide (TFM-10), 2-methoxy-4-bromoanilide (TFM-11), 2-methoxyl-4-chloro-5-methylaniline (TFM-12)] or bulkier structures such as 8-aminoquinoline (TFM-13), 5,6,7,8,-tetrahydro-1-naphthalenamine (TFM-14) and 4-bromo-naphthalenamide (TFM-15) had higher IC_{50} values compared with TFM. The IC_{50} values of TFM-10 and TFM-15 were >80 μM, indicating a loss of efficacy in these derivatives. Furthermore, structurally similar derivatives, in which the trifluoromethyl group was substituted by a methyl group (MET-01 or MET-09), failed to produce cytotoxic activity (IC_{50} >80 μM). These results indicate that the trifluoromethyl group at the C_5-carbon of TFM and its derivatives is essential for their cytotoxicity.

To determine whether α-keto acids generated by the release of trifluoromethane thiol from the TFM derivatives is responsible for their cytotoxic activity, OB-01 and OB-09, which were predicted products of TFM-01 and TFM-09, respectively, were evaluated. Neither OB-01 nor OB-09 showed cytotoxic activity (IC_{50} >80 μM). These results confirmed that the trifluoromethyl group is responsible for the cytotoxicity of TFM and its derivatives [7–9].

To assess whether TFM derivatives were selective towards *E. histolytica*, the efficacy of TFM, TFM-01 and TFM-02 against a representative mammalian cell line was examined. The IC_{50} values of TFM, TFM-01 and TFM-02 against CHO cells were more than 100-times higher than for *E. histolytica* (709 ± 172, 982 ± 174 and 878 ± 149 μM, respectively), whilst those of difluoroanilide and dimethoxyanilide derivatives were higher or slightly lower (>1 mM and 554 ± 94 μM, respectively). It was also previously reported that a high concentration of TFM (100 μg/mL, corresponding to 493 μM) inhibited the growth of mouse myeloma cells [8]. Taken together, TFM and its derivatives have good selectivity toward *E. histolytica*.

To determine whether the observed increase in the cytotoxic effects of the amidated TFM derivatives compared with TFM was due to their higher degradation efficiencies by MGL, time kinetics of the degradation of TFM, TFM-01 and TFM-02 by two recombinant MGL proteins (MGL1 and MGL2) were investigated [5]. Trifluoromethane thiol generated from TFM by MGL1 or MGL2 increased with incubation time (Fig. 1). Degradation of TFM by MGL2 was ca. 12.5-fold faster compared with MGL1 at 10 min after incubation, which is also consistent with a previous study [5]. In contrast, decomposition of TFM-01 and TFM-02 by MGL1 and of TFM-02 by MGL2 was negligible, whilst that of TFM-01 by MGL2 was ca. 70% slower compared with TFM.

We further examined the degradation process of TFM-01 in the amoeba by directly measuring the products formed when

Table 1
Structures and IC_{50} values of TFM and its derivatives against *E. histolytica* in vitro.

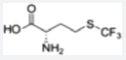
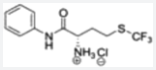
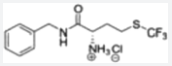
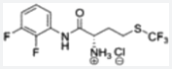
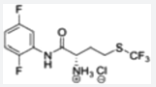
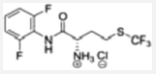
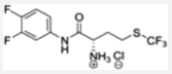
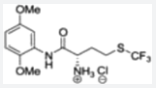
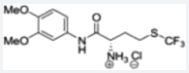
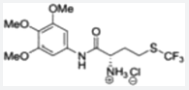
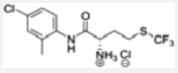
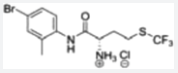
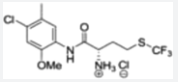
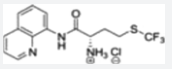
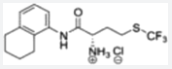
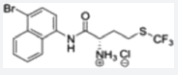
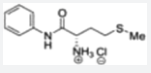
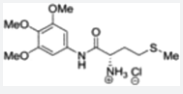
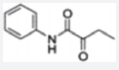
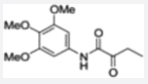
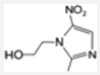
Compound name	Structure	IC_{50} (μM) (mean ± S.D.)
TFM		7.34 ± 0.59
TFM-01		2.22* ± 0.02
TFM-02		2.28* ± 0.05
TFM-03		2.37* ± 0.01
TFM-04		2.97* ± 0.02
TFM-05		2.46* ± 0.01
TFM-06		6.66* ± 0.16
TFM-07		1.11* ± 0.02
TFM-08		1.19* ± 0.01
TFM-09		1.28* ± 0.05
TFM-10		>80
TFM-11		16.86* ± 0.54
TFM-12		10.02 ± 0.06
TFM-13		12.48 ± 0.02
TFM-14		11.58 ± 0.17
TFM-15		>80
MET-01		>80
MET-09		>80
OB-01		>80
OB-09		>80

Table 1 (*Continued*)

Compound name	Structure	IC_{50} (μM) (mean ± S.D.)
MNZ		4.76* ± 0.22

* *P* values <0.05 as compared to TFM.

TFM-01 was mixed with the amoebic cell lysate. TFM, TFM-01 and OB-01 were unequivocally quantified on CE-TOFMS (Fig. 2). TFM was detected in the reaction mixture at 10 min and later after the parasite lysate was mixed with TFM-01 (Fig. 2B, solid line). The concentration of TFM detected corresponded to ca. 1% of the initial TFM-01 concentration (2 mM) and did not change after further incubation. The 2-oxobutyrate detected in the reaction mixture increased during the incubation time in a linear fashion, suggestive of continuous hydrolysis and decomposition of TFM-01 that leads to 2-oxobutyrate formation (Fig. 2B, broken line). Neither TFM nor 2-oxobutyrate was detected when the cell lysate was mixed with OB-01 (data not shown). The TFM derivatives containing piperidine, pyrrolidine or morpholine linked to the C_1-carbon (without the amide bond) showed no amoebicidal activity (data not shown). These data suggest that hydrolysis of the TFM derivatives is essential for amoebicidal activity.

To confirm whether the peptidases are involved in the hydrolysis of TFM-01, the cell lysate was pre-incubated with 0.1 mM *N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E-64) or 10 mM ethylene diamine tetra-acetic acid (EDTA), respectively. These protease inhibitors reduced decomposition of TFM-01 by ca. 60% and 35%, respectively (data not shown),

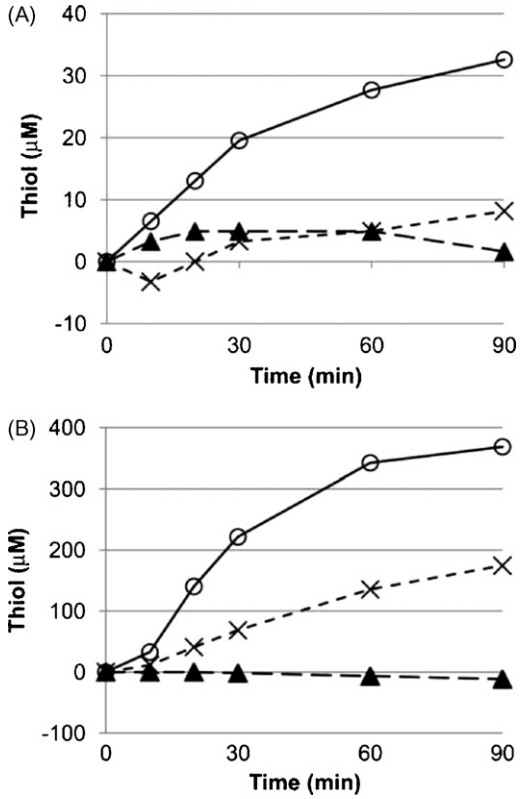


Fig. 1. Time kinetics of the degradation of trifluoromethionine (TFM) and two derivatives [anilide (TFM-01) and benzylamide (TFM-02)] by recombinant L-methionine γ-lyase (MGL). TFM (○), TFM-01 (×) and TFM-02 (▲) were incubated with (A) MGL1 or (B) MGL2 and released thiols were measured as described in Section 2.4.

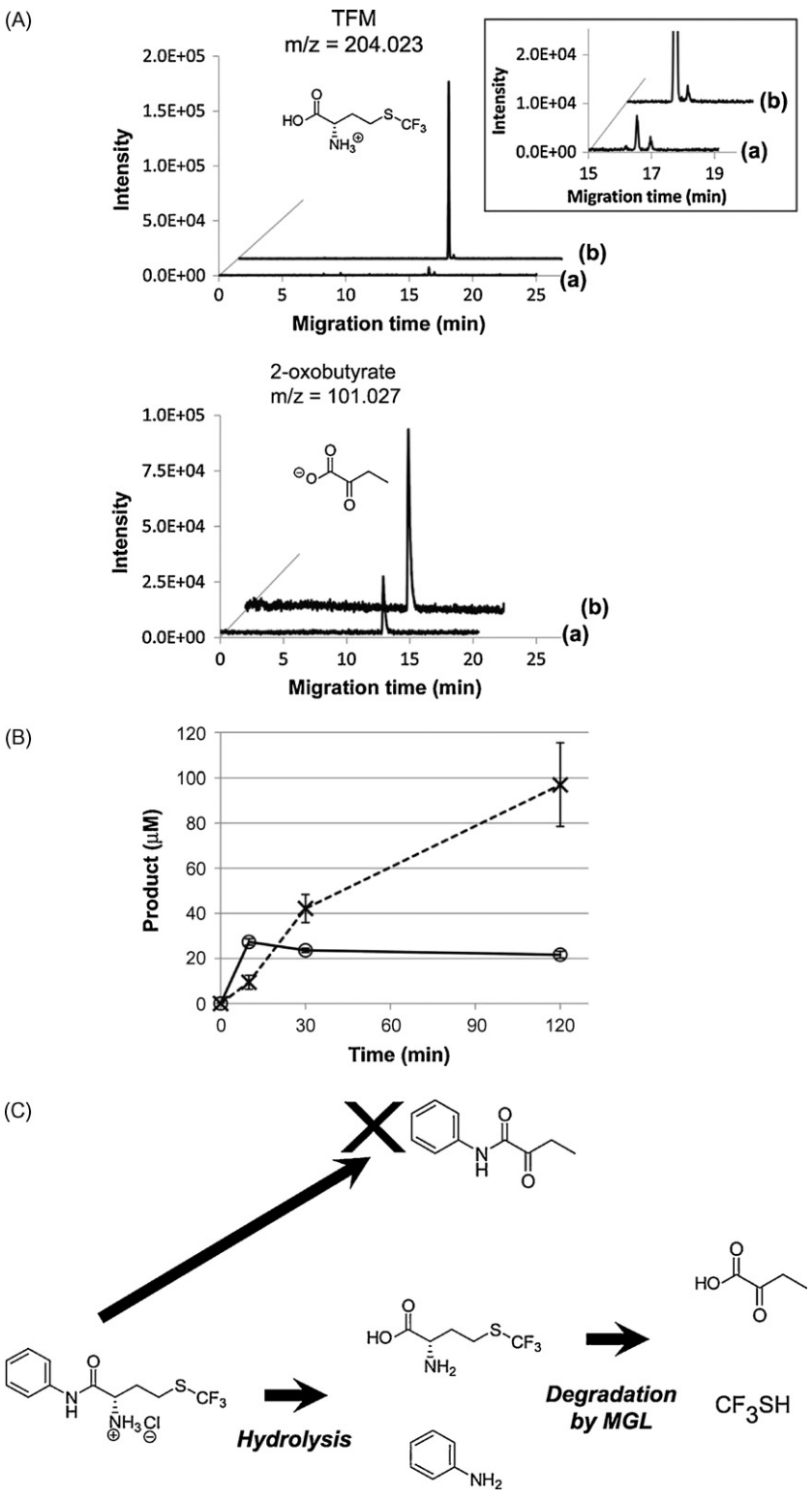


Fig. 2. Time kinetics of trifluoromethionine (TFM) derivative degradation by the amoebic cell lysate. (A) Identification and quantification of TFM and 2-oxobutyrate. Representative electropherograms of TFM (upper panel; a magnified figure is also shown in the inset) and 2-oxobutyrate (lower panel) of TFM-01 incubated with (a) the cell lysate at 37 °C for 2 h and (b) the standard (100 μM). (B) The amoebic cell lysate (50 μg) was incubated with 2 mM TFM-01 or 5% dimethyl sulphoxide (DMSO) (control) for the indicated times. The concentration of TFM (○) and 2-oxobutyrate (×) was quantified by capillary electrophoresis with electrospray ionisation time-of-flight mass spectrometry (CE-TOFMS). Data shown are mean ± standard deviation in triplicate. (C) A proposed scheme for TFM-01 degradation in *Entamoeba histolytica*. MGL, L-methionine γ-lyase.

suggesting that cysteine proteases and metalloproteases participate in the hydrolysis of TFM derivatives in *E. histolytica*. It is conceivable to assume that TFM is incorporated into the amoebae by amino acid transporter(s). It has also been reported that the parasite incorporates amino acids directly from the culture medium [12]. However, it remains to be clarified whether the TFM derivatives are incorporated via the presumed amino acid transporter(s) or by passive diffusion due to their hydrophobic nature.

The amoebicidal activity of the 16 compounds containing the trifluoromethyl group was evaluated using a hamster liver

Table 2
Amoebicidal activity of trifluoromethionine (TFM) derivatives by subcutaneous administration in a hamster amoebic liver abscess model.

Compound name	Increase in body weight (%) (mean ± S.E.)[a]	Weight of abscess (%) (mean ± S.E.)[b]	Number of animals
TFM	22.9 ± 12.8	24.5 ± 14.8	3
TFM-01	13.4 ± 0.5	29.1 ± 5.2	2
TFM-02	41.2 ± 4.3	17.9 ± 1.1	3
TFM-03	26.6 ± 2.9	32.6 ± 1.7	3
TFM-04	27.7 ± 3.4	30.3 ± 7.5	3
TFM-05	25.1 ± 2.8	15.4 ± 5.1	3
TFM-06	45.8 ± 10.6	17.7 ± 0.8	3
TFM-07	30.9 ± 2.6	15.3 ± 5.6	2
TFM-08	19.1 ± 7.2	0.2 ± 0.2	3
TFM-09	4.3 ± 4.3	8.2 ± 7.9	3
TFM-10	7.9 ± 7.9	0.2 ± 0.1	3
TFM-11	0.4 ± 0.2	19.4 ± 5.6	3
TFM-12	0.2 ± 0.2	3.2 ± 1.5	3
TFM-13	26.7 ± 13.6	5.4 ± 5.2	3
TFM-14	24.6 ± 6.3	5.6 ± 5.6	3
TFM-15	3.5 ± 0.8	0.0 ± 0.0	3
Control[c]	26.5 ± 9.0	39.7 ± 1.8	3

[a] Percentage increase in body weight between Days 0 and 6.
[b] Percentage of the abscess relative to the weight of the liver.
[c] DMSO (100 μL/head).

abscess model (Table 2). Trophozoites were directly injected into the liver and 24 h later 0.2 μmol of TFM or its derivatives were subcutaneously injected into the hamsters. Two difluoroanilide derivatives (TFM-04 and TFM-05), one dimethoxyanilide (TFM-07) and 5,6,7,8-tetrahydro-1-naphthylamide compound (TFM-14) exhibited antiamoebic effects comparable with that of TFM. All the other compounds except for TFM-06, TFM-09, TFM-11 and TFM-15 also showed comparable or slightly lower efficacy compared with TFM. It is also worth noting that the hamsters treated with TFM-04, TFM-07 or TFM-14 gained more weight compared with the other effective compounds.

Next we evaluated the in vivo efficacy of TFM-14, which showed the highest efficacy by subcutaneous administration, and TFM via oral administration (Table 3). Single oral administration of TFM at 2.54 mg/kg (0.5 μmol/animal equivalent) completely prevented liver abscess formation; TFM-14 had comparable or slightly lower potency compared with TFM [100% cure with 9.22 mg/kg (1.0 μmol/animal equivalent)]. Pargal et al. [13] showed that only 19 of 29 animals receiving 100 mg/kg (ca. 30 μmol/animal) oral metronidazole were cured of amoebic liver abscesses. Although we may not be able to compare directly their data [13] with ours because of subtle differences in experimental conditions, TFM and TFM-14 are likely to be more effective in preventing the formation of amoebic liver abscesses than metronidazole in the hamster model.

Table 3
Amoebicidal activity of trifluoromethionine (TFM) derivatives by oral administration in a hamster amoebic liver abscess model.

Compound name	Dose (mg/kg)	Increase in body weight (%) (mean ± S.E.)[a]	Weight of abscess (%) (mean ± S.E.)[b]	Number of animals
TFM	1.02	35.1 ± 2.1	7.8 ± 5.9	3
	2.54	27.6 ± 4.5	0.0 ± 0.0	3
	3.56	34.5 ± 2.2	1.7 ± 1.4	3
	5.08	27.7 ± 5.9	0.0 ± 0.0	3
TFM-14	1.84	26.3 ± 3.2	30.2 ± 1.7	3
	4.61	23.3 ± 8.3	11.3 ± 9.2	3
	6.45	22.4 ± 2.9	3.9 ± 2.5	3
	9.22	33.9 ± 3.7	0.0 ± 0.0	3
Control[c]		5.0 ± 0.9	47.6 ± 0.0	2

[a] Percentage increase in body weight between Days 0 and 6.
[b] Percentage of the abscess relative to the weight of the liver.
[c] DMSO (100 μL/head).

We have previously shown that the carbonothionic difluoride derived from TFM degradation by MGL non-specifically cross-links the primary amino group of MGL and other proteins in vitro and is responsible for the cytotoxicity of TFM [5]. Since the target of carbonothionic difluoride is non-specific and is not limited to the targets of 5-nitroimidazoles, such as pyruvate:ferredoxin oxidoreductase, thioredoxin reductase and nitroreductase [14–16], TFM and its derivatives are expected to be effective against 5′-nitroimidazole-resistant *E. histolytica* [17], *T. vaginalis* [18], periodontal bacteria and *Citrobacter freundii*.

Acknowledgments

The authors thank Rumiko Kosugi (Department of Parasitology, Gunma University Graduate School of Medicine, Gunma, Japan) for technical assistance. They also thank Mr Yuji Kakazu and Mr Akiyoshi Hirayama (Institute for Advanced Biosciences, Keio University, Yamagata, Japan) for technical help with mass spectrometry.

Funding: This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to DS (20590429) and TN (18GS0314, 18050006 and 18073001), a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan, and a grant for research to promote the development of anti-AIDS pharmaceuticals from the Japan Health Sciences Foundation to TN.

Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2009.08.016.

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