*J Antimicrob Chemother* 2015; **70**: 3061–3069
doi:10.1093/jac/dkv231 Advance Access publication 18 August 2015

Journal of Antimicrobial Chemotherapy

# Gastrointestinal localization of metronidazole by a lactobacilli-inspired tetramic acid motif improves treatment outcomes in the hamster model of *Clostridium difficile* infection

**Philip T. Cherian[1]†, Xiaoqian Wu[2,3]†, Lei Yang[1], Jerrod S. Scarborough[1], Aman P. Singh[1,4], Zahidul A. Alam[2], Richard E. Lee[1]‡ and Julian G. Hurdle[2,3,5]*‡**

*[1]Department of Chemical Biology and Therapeutics, St Jude Children's Research Hospital, Memphis, TN 38105, USA; [2]Department of Biology, University of Texas, Arlington, TX 76019, USA; [3]Center for Infectious and Inflammatory Diseases, Institute of Biosciences and Technology, Texas A&M Health Science Center, Houston, TX 77030, USA; [4]Biomedical Sciences Program, Graduate Health Sciences, University of Tennessee Health Science Center, Memphis, TN 38163, USA; [5]Department of Microbial and Molecular Pathogenesis, Texas A&M Health Science Center, College of Medicine, Bryan, TX 77807, USA*

*Corresponding author. E-mail: jhurdle@ibt.tamhsc.edu
†These authors contributed equally to experiments.
‡These authors conceived, designed and supervised the study.

*Received 2 March 2015; returned 8 May 2015; revised 29 June 2015; accepted 7 July 2015*

**Objectives:** Metronidazole, a mainstay treatment for *Clostridium difficile* infection (CDI), is often ineffective for severe CDI. Whilst this is thought to arise from suboptimal levels of metronidazole in the colon due to rapid absorption, empirical validation is lacking. In contrast, reutericyclin, an antibacterial tetramic acid from *Lactobacillus reuteri*, concentrates in the gastrointestinal tract. In this study, we modified metronidazole with reutericyclin's tetramic acid motif to obtain non-absorbed compounds, enabling assessment of the impact of pharmacokinetics on treatment outcomes.

**Methods:** A series of metronidazole-bearing tetramic acid substituents were synthesized and evaluated in terms of anti-*C. difficile* activities, gastric permeability, *in vivo* pharmacokinetics, efficacy in the hamster model of CDI and mode of action.

**Results:** Most compounds were absorbed less than metronidazole in cell-based Caco-2 permeability assays. In hamsters, lead compounds compartmentalized in the colon rather than the bloodstream with negligible levels detected in the blood, in direct contrast with metronidazole, which was rapidly absorbed into the blood and was undetectable in caecum. Accordingly, four leads were more efficacious ($P<0.05$) than metronidazole in *C. difficile*-infected animals. Improved efficacy was not due to an alternative mode of action, as the leads retained the mode of action of metronidazole.

**Conclusions:** This study provides the clearest empirical evidence that the high absorption of metronidazole lowers treatment outcomes for CDI and suggests a role for the tetramic acid motif for colon-specific drug delivery. This approach also has the potential to lower systemic toxicity and drug interactions of nitroheterocyclic drugs for treating gastrointestine-specific diseases.

## Introduction

The gastrointestinal (GI) tract is the primary disease site for *Clostridium difficile* infection (CDI), which is the leading cause of hospital-acquired diarrhoea in developed countries. Clinical manifestations of CDI range from mild-to-moderate diarrhoea to severe colitis and toxic megacolon,[1] which is mediated by the production of toxins TcdA and TcdB, which are responsible for tissue inflammation and epithelial damage.[1] The antibiotics metronidazole and vancomycin are the current first-line treatments for mild and severe forms of CDI, respectively.[1] Traditionally, metronidazole was the preferred choice of treatment for CDI, owing to its low cost and being as effective as vancomycin in treating mild-to-moderate CDI in most patients.[2,3] However, this treatment paradigm has changed in the setting of severe CDI,[4] as best exemplified by a recent clinical report where the overall clinical success with metronidazole was 72.7% compared with 81.1% for vancomycin treatment.[4] Whilst metronidazole is more potent than vancomycin *in vitro*, its poorer efficacy for the treatment of severe CDI is thought to arise from the drug being highly absorbed

from the upper GI tract, with only low levels of drug (6%–15%) occurring in the colon after administration.[3,5] In contrast, vancomycin is non-absorbed, achieving high concentrations in the colon that are at least two orders of magnitude higher than those of metronidazole.[3,5] Nonetheless, there is a lack of empirical studies on the impact of metronidazole's pharmacokinetics on treatment outcomes for CDI, in either animal models or in CDI patients. We reasoned that strategies localizing metronidazole to the GI tract, bolstering its concentration in the lower bowel, could improve treatment outcomes for CDI by taking advantage of its bactericidal activity against *C. difficile*.

To localize metronidazole to the GI tract, we hypothesized that chemical modification of metronidazole with a poorly permeable tetramic acid moiety[6] could decrease absorption and introduce novel mode of action properties associated with tetramic acids. Tetramic acids comprise a large family of pharmacologically active natural products, which show narrow-spectrum antibacterial activities that are often restricted to Gram-positive bacteria.[7] In general, the modes of action of representative tetramic acids arise from inhibition of bacterial cell wall biosynthesis,[8] DNA topoisomerases,[9] RNA polymerase activity[10] or dissipation of the membrane potential.[11] Recently, we described that the tetramic acid reutericyclin, which is produced by some strains of *Lactobacillus reuteri*, effectively kills *C. difficile* by disrupting the membrane potential.[6,12] Similarly, other acyltetramic acids also inhibit the growth of *C. difficile*.[13] Interestingly, reutericyclin and closely related analogues were minimally absorbed in the Caco-2 intestinal permeability model.[6] Thus, we applied this pharmacophore to metronidazole, obtaining minimally absorbed derivatives with better efficacy than the unmodified drug. This now provides direct evidence for the role played by the pharmacokinetics of metronidazole in influencing treatment outcomes for CDI.

## Materials and methods

### *Synthesis of compounds*

Complete details of the synthesis procedures and compound characterization for the derivatization of metronidazole with tetramic acid moiety are described in the Supplementary data (available at *JAC* Online). Briefly, for the synthesis of **1971** and similar metronidazole–tetramic acid analogues, the alcohol of metronidazole was displaced by nosylated amino acid esters using the Fukuyama–Mitsunobu amination protocol.[14] Following removal of the nosyl group,[15] the free secondary amine was acylated with a ketene–acetone adduct and the intermediate cyclized using Lacey–Dieckmann conditions.[16,17] The final mixtures were purified by reverse-phase column chromatography (RPCC) to provide the metronidazole–tetramic acid hybrids in 19%–65% overall yields. To synthesize **2122**, the alcohol of metronidazole was converted into the amine under Mitsunobu conditions[18] while the 3-methoxycarbonyl tetramic acid was synthesized from Leu-OMe hydrochloric acid and methyl malonyl chloride using Lacey–Dieckmann conditions. Reaction of these two intermediates in a microwave[19] at 100°C for 10 min followed by purification by RPCC provided **2122** in 39% yield. For **2123**, the alcohol of metronidazole was oxidized to acid by Jones oxidation[20] while the tetramic acid was synthesized from Z-Leu-OH and (triphenylphosphoranylidene)ketene using the procedure described by Schobert *et al*.[21] Finally, the two intermediates were coupled in presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 4-dimethylaminopyridine and purified by RPCC to provide **2123** in 58% yield.[22] The final compounds were characterized by LC-MS and $^{13}$C- and $^{1}$H-NMR. All reported compounds were ≥95% pure.

### *Determination of growth inhibitory and bactericidal concentrations against C. difficile*

The *C. difficile* strains R20291 (ribotype 027, from A. Sonenshein, Tufts University, USA) and BAA-1875 (ribotype 078, from ATCC) were used for most *in vitro* assays. MICs of compounds were evaluated as described previously[23] in brain heart infusion (BHI) broth in 96-well microtitre plates, with a bacterial inoculum of ~$10^6$ cfu/mL. The MIC was defined as the lowest concentration of compound inhibiting visible growth after 24 h of incubation in an A35 anaerobic chamber (Don Whitley Scientific). MBCs were determined for logarithmic-phase and stationary-phase cultures in BHI broth in 24-well plates.[23] The MBC was defined as the lowest concentration of compound causing ≥3 log reduction in the initial cell inoculum (~$10^7$ cfu/mL) after 24 h. All MIC and MBC measurements were performed at least twice.

### *Caco-2 cell permeability assay*

The Caco-2 cell permeability assay was carried out as described previously.[24]

### *Animal experiments*

All animal experiments reported herein were approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington. Animal experiments were done in accordance with the University Standard Operating Procedures, which adhere to the regulations outlined in the USDA Animal Welfare Act (9 CFR, Parts 1–3).

#### *Hamster model of CDI*

Golden Syrian hamsters (~100 g) from Charles River Laboratories were separately housed in sterile cages and maintained on sterile food and water. On day −1, animals were subcutaneously injected with clindamycin phosphate solution (50 mg/kg; Hospira). After 20 h (day 0), hamsters were infected by oral gavage with $10^6$ cfu of the *C. difficile* strain ATCC 43596 that was grown in sporulation medium and washed once with pre-reduced PBS;[12] the average number of spores in the diluted inocula was 2.3 log. ATCC 43596 is a metronidazole-susceptible toxigenic strain that is highly virulent in the hamster model of CDI. From days 1 to 5, hamsters ($n \geq 8$ per group) were treated once daily with vehicle (PEG-400:water, 85:15) or 50 mg/kg of test compounds or metronidazole in vehicle. Vancomycin (20 mg/kg) was used as a control. After 5 days of treatment, surviving hamsters were monitored for up to 30 days for signs of disease as described by Anton *et al*.[25] All moribund animals were euthanized as well as those that survived the post-treatment monitoring period (30 days); caeca were recovered from all animals.

#### *Pharmacokinetics in hamsters*

(i) ***Plasma concentration*** Pharmacokinetic studies were assessed in male Syrian hamsters (~100 g), from Charles River, with each carrying a pre-implanted jugular vein cannula. Hamsters ($n=5$ per group) were fasted overnight and for the duration of the experiment (7 h). After collecting pre-dose blood samples (200 μL), animals were dosed with 100 mg/kg of compounds formulated in PEG-400:water (85:15). At various timepoints, blood samples were collected into heparin-coated tubes that were centrifuged at 3000 rpm for 10 min to yield plasma, which was stored at −20°C.

(ii) ***Caecal concentration*** After dosing animals ($n=3$ per timepoint) as above, animals were humanely sacrificed at timepoints and their caecal contents collected and stored at −20°C.

(iii) ***LC-MS*** For plasma samples, 25 μL of plasma was placed in a 384-well analytical plate and quenched by the addition of 50 μL of acetonitrile

containing 4 mg/L warfarin as internal standard. The plate was sealed, shaken at 600 rpm for 10 min and then centrifuged at 4000 rpm for 20 min. Next, 15 μL of the supernatant was transferred to a new analytical 96-well plate and mixed with 100 μL of MilliQ water. The samples were analysed by injecting 5 μL onto a Waters UPLC/SQD LC-MS/MS system. The caecal samples were processed by adding 100 μL of acetonitrile containing 4 mg/L warfarin (internal standard) to the microfuge tubes containing caecal matter (~50 mg). The suspension was vortexed for 10 s, sonicated for 1 min and then centrifuged at 10000 rpm for 10 min. Aliquots (50 μL) of the collected supernatants were then transferred to a 384-well plate and analysed by LC-UV-HRMS using Waters AQUITY UPLC and Waters XEVO G2 QTOF mass spectrometer.

(iv) ***Effect of caecal contents on compound activity*** The MICs of test compounds following exposure to caecal material (20% w/v) from drug-free hamsters were determined as previously described,[12] except against ATCC 43596 in BHI broth.

### *Antimicrobial assessment*

Selected lead compounds were further evaluated in terms of their spectrum of activity against representative intestinal anaerobes and various clinical strains of *C. difficile* by agar dilution in Wilkins–Chalgren agar.[23] Representative intestinal anaerobes were from BEI Resources and clinical *C. difficile* were from various sources. Activities against metronidazole-resistant *C. difficile* were measured in Brucella agar containing haemin (5 mg/L), vitamin K1 (1 mg/L) and sheep blood (5%).[12] Effects on cell viability and the transcriptional responses of cells to inhibition were determined as described previously.[12,26] Further details are provided in the Supplementary data.

## Results

### *Discovery of metronidazole–tetramic acid hybrids*

To ensure that hybridization of metronidazole to tetramic acid did not eliminate activity against *C. difficile*, we first determined the optimal linker strategy to join metronidazole to the tetramic acid moiety. Relying on prior literature that the alcohol portion of metronidazole can be modified without affecting its activity,[27] we reasoned the tetramic acid moiety could be introduced at the alcohol position. Then, we determined which position on the tetramic ring would be the ideal attachment site for metronidazole. Based on our previous studies,[11] synthetic feasibility and availability of starting materials, we modified the N1 and C3 positions of the tetramic acid core (Figure 1). This led to three analogues: the N1-alkyl **1971**, the C3-carboxamide **2122** and the C3-acyl **2123** (Supplementary data, Schemes 1, 2 and 3). MIC testing (Table 1) revealed that metronidazole linked to tetramic acid at the N1 position, as in **1971** (MIC = 1–2 mg/L), was optimal for producing molecules that retain activity against *C. difficile*. The C3-linked analogues (**2122** and **2123**) were much less active (24- and 10-fold less active than **1971**, respectively). Although **1971** was 4–8-fold less active than metronidazole (MIC = 0.25 mg/L), this did not diminish further expansion of the compound series, since lower activity could be compensated for *in vivo* by increased local concentrations of drugs.

### *Structure–activity relationships (SAR) of metronidazole–tetramic hybrids*

Expansion of **1971** compound series was achieved through SAR studies. We opted to expand the SAR using amino acid R-group functionalities to cover a range of physicochemical properties, such as hydrophobicity, polarity and charge, that could influence absorption from the intestinal tract.[28] This led to the generation of a library of compounds with a variety of functional groups at the 5-position (Supplementary data, Scheme 1). The results of the SAR are shown in Table 2. Substitution at the 5-position of the tetramic core was an important variant for activity, as the derivative lacking a 5-substituent (**2153**, R = H) was >64-fold less active than parent **1971**. Amongst the various substituents, the hydrophobic substituents were preferred, as polar and charged substituents led to significant loss of activity as demonstrated by comparing the activities of masked and unmasked pairs of aliphatic alcohols—**2171/72**, **2173/74** and **2124/25**, carboxylic acids—**2175/76** and **2177/78** and amines—**2179/80** and **2309/10**. The lack of activity of these polar analogues is most likely due to poor membrane partition, resulting in low intracellular levels.[11] In the case of the hydrophobic substituents, both aliphatic and aromatic groups were tolerated and their activities were generally comparable to **1971** (Table 1). As the tetramic acid motif is present in several cytotoxic natural products,[7] we examined whether they augmented the cytotoxicity of metronidazole. They did not show elevated cytotoxicity against Vero epithelial kidney cells (ATCC CCL-81; Table S1). Thus, the SAR study provided several active analogues, in addition to **1971**, that were subjected to further analysis as described below.

### *Intestinal cell line permeability studies*

To rapidly evaluate whether metronidazole–tetramic acid hybrids displayed poor absorption from the apical side of the GI tract, we deployed the Caco-2 cell permeability assay that provides good prediction of compound intestinal absorption.[29] The permeability coefficient ($P_{app}$ A-B) of the compounds was calculated (Table S2) and the ability of compounds to move from the apical to the basolateral side of the Caco-2 monolayer is

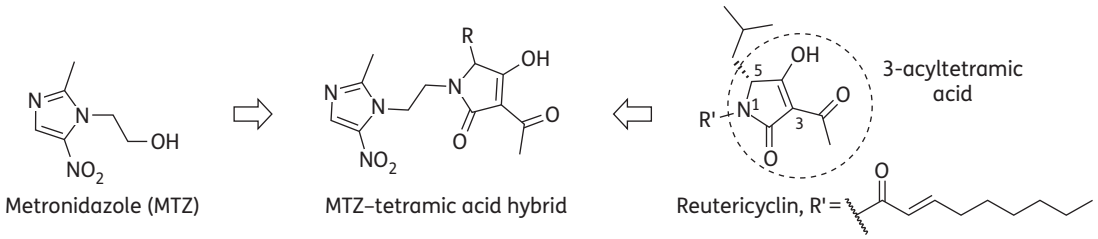
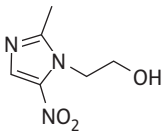
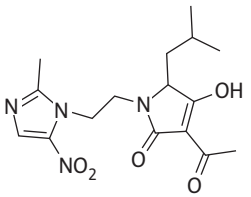
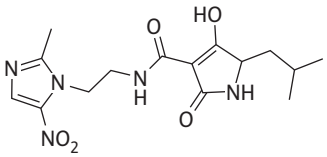
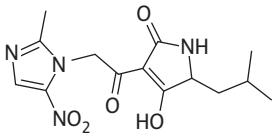



**Figure 1.** Overall strategy of hybridizing metronidazole to the tetramic acid motif in reutericyclin.

**Table 1.** Design and activities of metronidazole–tetramic acid hybrids

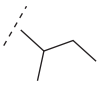
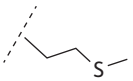
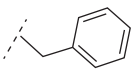
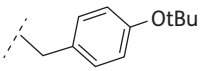
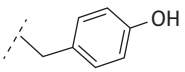
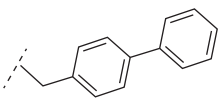
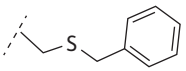
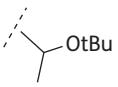
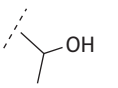
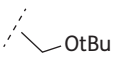
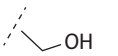
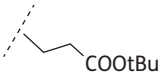
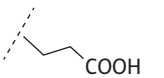
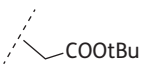
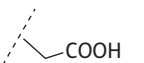
| Compound | Structure | MIC (mg/L) R20291 | MIC (mg/L) BAA-1875 |
|---|---|---|---|
| vancomycin | — | 2 | 0.75 |
| metronidazole |  | 0.125 | 0.25 |
| **1971** |  | 1 | 2 |
| **2122** |  | 24 | 32 |
| **2123** |  | 12 | 20 |

shown in Figure 2. As expected, the unmodified metronidazole (480.1 ± 54.8 nm/s) was highly permeable, while the minimally absorbed vancomycin (214.8 ± 2.7 nm/s) displayed poor permeation across the Caco-2 monolayer. In contrast to metronidazole, all tetramic acid hybrids (range 176.9 ± 14.3 to 358.5 ± 12 nm/s) displayed poorer permeation from the apical to the basolateral side of cells, implying they are likely to be compartmentalized in the lumen of the GI tract. Compounds **2154**, **2155** and **2313** (range 284.5 ± 34.7 to 307 ± 35.6 nm/s) were slightly more permeable than vancomycin, suggesting that within the compound panel there are derivatives that may have some limited permeability, which could be suitable for treating infections residing in intracellular niches.[30]

### *Efficacy and pharmacokinetic studies in hamsters*

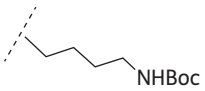
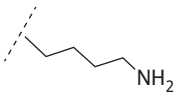
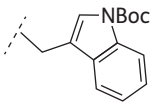
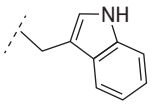
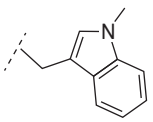
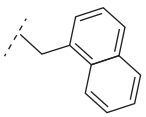
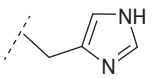
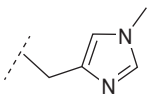
The gold-standard hamster model of CDI was adopted to investigate whether decreased permeability for metronidazole could lead to improved efficacy. Therefore, four lead compounds showing good activity, decreased permeability, and which covered a diverse array of substitutions at the 5-position of the tetramic core (**1971**—isobutyl, **2345**—biphenyl, **2344**—naphthyl and **2490**—n-methyl indole) were compared with metronidazole at 50 mg/kg and the results are shown in Figure 3. During efficacy experiments, animals showed no signs

**Table 2.** Structure–activity relationship of metronidazole–tetramic acid hybrids

| Compound | Structure | MIC (mg/L) R20291 | MIC (mg/L) BAA-1875 |
|---|---|---|---|
| **2153** | H- | >64 | >64 |
| **2158** | $CH_3$- | 4 | 8 |
| **2156** |  | 1 | 2 |
| **2155** |  | 0.25 | 1 |
| **2157** |  | 8 | 16 |
| **2154** |  | 4 | 4 |
| **2171** |  | 0.5 | 2 |
| **2172** |  | >64 | >64 |
| **2345** |  | 0.5 | 0.25 |
| **2313** |  | 2 | 1.5 |
| **2173** |  | 1 | 2 |
| **2174** |  | >64 | >64 |
| **2124** |  | 2 | 3 |
| **2125** |  | >64 | >64 |
| **2175** |  | 2 | 4 |
| **2176** |  | >64 | >64 |
| **2177** |  | 8 | 16 |
| **2178** |  | >64 | >64 |

*Continued*

**Table 2.** *Continued*

| Compound | Structure | MIC (mg/L) R20291 | MIC (mg/L) BAA-1875 |
|---|---|---|---|
| **2179** |  | 8 | 16 |
| **2180** |  | >64 | >64 |
| **2309** |  | 1.5 | 0.75 |
| **2310** |  | 6 | 16 |
| **2490** |  | 1 | 1.5 |
| **2344** |  | 1 | 1 |
| **2312** |  | >64 | >64 |
| **2489** |  | >64 | >64 |

of treatment-related adverse effects, with treatment outcomes statistically ($P<0.05$) superior to metronidazole in an acute form of CDI in hamsters. Animals treated with metronidazole survived a maximum of 10 days post-infection, whilst treatment with the hybrids improved their survival by an additional 1–5 days. However, the compounds did not provide complete survival for >15 days post-infection. In contrast, animals treated with vancomycin (20 mg/kg) became moribund within 18–28 days post-infection (Figure S1). The differences in efficacies between metronidazole and the hybrids were not due to improved activities of the hybrids compared with metronidazole against the infecting strain ATCC 43596 as their MICs were all similar: 0.125 mg/L for metronidazole, 0.50 mg/L for **1971** and 0.25 mg/L for **2344**, **2345** and **2490**. Similarly, the increased lipophilicity of the compounds compared with metronidazole did not affect the activity of the compounds when exposed to caecal contents (20% w/v; Table S3).

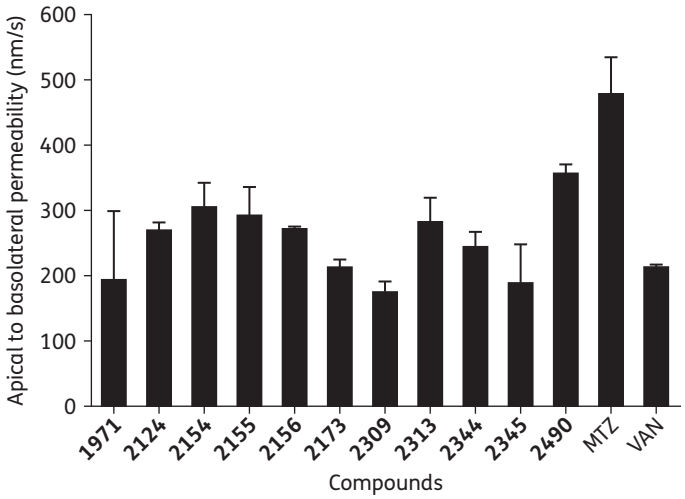



**Figure 2.** Gastrointestinal absorption of compounds from the apical to basolateral side of a Caco-2 monolayer. MTZ, metronidazole; VAN, vancomycin.

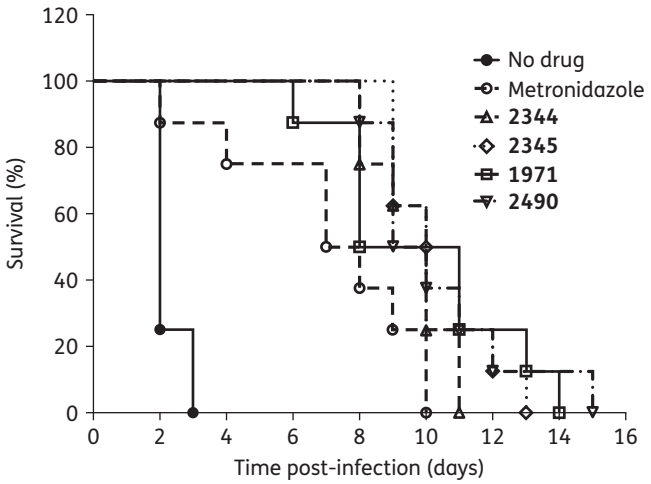



**Figure 3.** Efficacy of metronidazole compared with metronidazole–tetramic acid hybrid compounds. Syrian hamsters ($n\geq8$ per group) were treated with drug at 50 mg/kg, once daily and monitored twice each day. Comparison of the survival curves shows statistical significance for all hybrids versus metronidazole at $P<0.05$.

In order to test whether the hybrids also exhibit poorer permeabilities than metronidazole across the GI tract, we determined the concentrations of **2344**, **2345** and metronidazole in plasma. A single 100 mg/kg dose of **2344**, **2345** or metronidazole in the pharmacokinetic model induced short-term diarrhoeal symptoms (wet tails) in ~50% of animals, which is a side effect of metronidazole (data not shown). As seen in Figure 4(a), the concentrations of the hybrids in plasma were much lower than metronidazole for both maximum concentration obtained [$C_{max}$ (mg/L): metronidazole, 51.04; **2344**, $2.11\times10^{-1}$; **2345**, $6.16\times10^{-1}$ and total exposure AUC (mg·h/L): metronidazole 135.29; **2344**, $3.86\times10^{-1}$; **2345**, $3.55\times10^{-1}$; Table S4]. The $T_{max}$ of **2344** and **2345** was 0.25 h and of metronidazole was 1 h (Table S4). *In vitro* ADME assays showed the compounds to be highly stable ($t_{1/2}>4.5$ h) in plasma

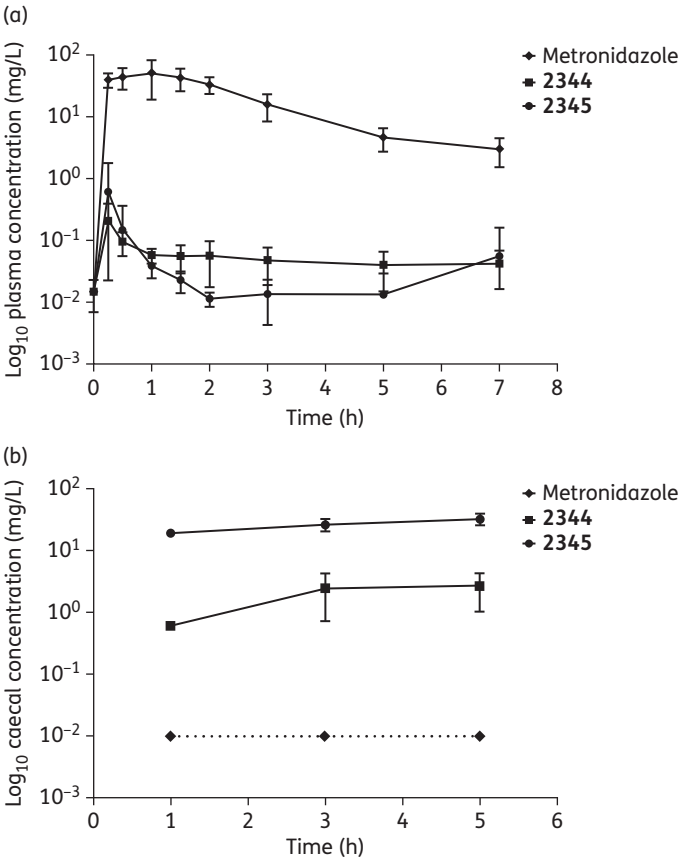



**Figure 4.** Differences in the pharmacokinetics of metronidazole and metronidazole–tetramic acid hybrids **2344** and **2345**, following a single dose of compound at 100 mg/kg. (a) Peak plasma concentrations: metronidazole, 51.04 mg/L; **2344**, $2.11\times10^{-1}$ mg/L; and **2345**, $6.16\times10^{-1}$ mg/L. (b) Peak caecal concentrations: **2345**, 32.65 mg/L; **2344**, 2.68 mg/L; and metronidazole, $<1.0\times10^{-2}$ mg/L (undetectable, LLOQ for the assay is shown).

(Table S5), with much higher serum protein binding than metronidazole (Table S6) and varying microsomal stability ($t_{1/2}$: **2344**, 0.30 h; **2345**, 1.33 h; Table S7). Thus, the very low concentration of the hybrids in the plasma might likely be due to a combination of poor intestinal absorption and hepatic clearance. To evaluate this *in vivo*, we determined the concentrations of the three compounds in the caecal contents recovered from hamsters at 1, 3 and 5 h timepoints following oral dosing (Figure 4b). The mean peak caecal concentrations of **2345** and **2344** were 32.65 mg/L and 2.68 mg/L, respectively, while metronidazole was not detected even at the $1.0\times10^{-2}$ mg/L lower limit of quantification (LLOQ) of the assay. Thus, these hybrids had better retention in the GI tract than metronidazole, which mimics the results from our Caco-2 cell permeability study. The 12-fold difference in the caecal concentration of **2344** and **2345** may reflect better solubility for **2345** in the PEG:water vehicle used (data not shown), since they both possess similar gastric stability profiles (Figure S2) and were also similar in their plasma stability and aqueous solubility at different pHs (Table S5 and Table S8).

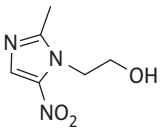
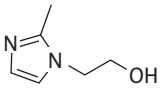
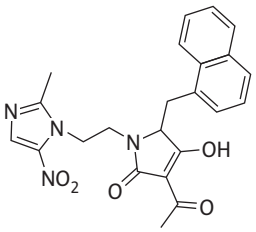
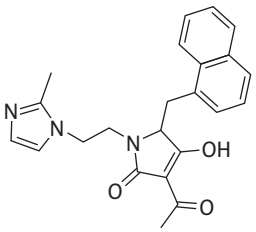
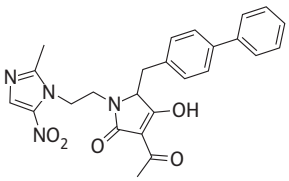
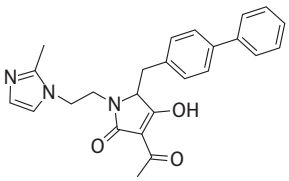
### *Anti-C. difficile properties of leads*

Across all tests, the four leads (**1971**, **2344**, **2345** and **2490**) demonstrated antimicrobial profiles that were similar to metronidazole, including: mean $MIC_{50}$ and $MIC_{90}$ against clinical isolates of *C. difficile*, **1971** (1, 1.5 mg/L), **2344** (0.5, 0.75 mg/L), **2345** (0.5, 1 mg/L), **2490** (1–2 mg/L) and metronidazole (0.25, 0.5 mg/L; Table S9); retention of bactericidal activities against both growing and non-growing cells (Figure S3 and Table S10); similar activities against gut flora (Table 3); and lack of propensity for *de novo* resistance (Table S11). These similarities led us to query whether the hybrids displayed a similar mode of action to metronidazole. Since the activity of metronidazole is attributed to its nitro group,[31,32] we synthesized and tested the des-nitro analogues **2699** and **2700** of **2344** and **2345**, respectively. As seen in Table 4, the des-nitro analogues were completely inactive

**Table 3.** Mean agar MICs of metronidazole–tetramic acid hybrids against a panel of gut flora bacteria

| | | Mean MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| Bacteria | Strain | **1971** | **2344** | **2345** | **2490** | metronidazole | vancomycin |
| *Actinomycetes viscosus* | HM238 | 64 | 128 | 32 | 256 | 32 | 0.5 |
| *Bacteroides eggerthii* | HM210 | 0.5 | 0.5 | 1 | 1 | 0.25 | 24 |
| *Bacteroides fragilis* | ATCC 25285 | 128 | 96 | 24 | 256 | >512 | 2 |
| *B. fragilis* | HM20 | 0.5 | 0.25 | 1 | 1.5 | 0.25 | 16 |
| *Bacteroides ovatus* | ATCC 8483 | 3 | 1.5 | 1 | 2 | 0.75 | 64 |
| *B. ovatus* | HM222 | 2 | 1.25 | 0.375 | 2 | 0.75 | 24 |
| *Bacteroides* sp. | HM18 | 0.5 | 0.5 | 1 | 1.5 | 0.5 | 96 |
| *Bacteroides* sp. | HM19 | 2 | 1.5 | 2 | 6 | 0.375 | 128 |
| *Bacteroides* sp. | HM23 | 1 | 1 | 1.25 | 2 | 0.375 | 96 |
| *Bacteroides* sp. | HM28 | 1.5 | 0.75 | 1.5 | 2 | 0.75 | 48 |
| *Lactobacillus crispatus* | HM421 | >512 | 256 | 64 | 384 | >512 | 0.5 |
| *Bifidobacterium* sp. | HM30 | 512 | 192 | 48 | 192 | >512 | 0.5 |
| *Fusobacterium nucleatum* | HM260 | 1 | 4 | 10 | 4 | 0.25 | >512 |
| *Fusobacterium periodonticum* | HM41 | 0.5 | 0.5 | 1.5 | 1.5 | <0.06 | 192 |
| *Lactobacillus johnsonii* | HM 643 | >512 | >512 | 192 | >512 | >512 | 6 |
| *Porphyromonas uenonis* | HM130 | 48 | 16 | 4 | 48 | 384 | 3 |

**Table 4.** Comparison of mechanism of action of nitro and des-nitro metronidazole and analogues

| Compound | Structure | MIC (mg/L) R20291 | MIC (mg/L) BAA-1875 |
|---|---|---|---|
| metronidazole |  | 0.125 | 0.5 |
| **2698** (des-nitro metronidazole) |  | >128 | >128 |
| **2344** |  | 1 | 1 |
| **2699** |  | >128 | >128 |
| **2345** |  | 0.5 | 0.25 |
| **2700** |  | >128 | >128 |

(MIC >128 mg/L), suggesting that the hybrids displayed the same mode of action as metronidazole, involving biochemical reduction of its nitro group to reactive species, which cause cellular damage.[26] Indeed, like metronidazole the hybrids imposed similar cellular stresses in *C. difficile*, which was evident from the up-regulation of thioredoxin-related genes (*trxA1/trxB1* and CDR20291_2024), *recA*-mediated DNA repair and the hybrid cluster protein that responds to nitrosative stress and protein damage (Figure S4).[26] Accordingly, the hybrids were inactive against several *C. difficile* clinical strains displaying stable resistance to metronidazole (Table S12). These findings indicate that the improved activity of these hybrids over metronidazole is related to their retention in the GI tract, as opposed to an alternative antibacterial mechanism of action.

## Discussion

For >30 years, metronidazole has been adopted as the first-line treatment for CDI, owing to significant cost savings and effectiveness in mild-to-moderate CDI.[1] Nitroheterocyclic prodrugs continue to represent attractive treatment approaches for anaerobic GI and systemic infections caused by protozoa and bacterial pathogens.[33] However, metronidazole and other members within this drug class are not typically developed to be retained in the GI tract for localized treatment.[34,35] Recent clinical findings by Johnson *et al.*[4] confirmed that there is a clear difference in treatment outcomes between metronidazole and vancomycin in severe CDI, which has long been speculated to arise from differences in their pharmacokinetic profiles. For the first time, our study now provides well-defined evidence that the poor distribution of metronidazole may hinder treatment outcomes. This was achieved by hybridizing metronidazole to a tetramic acid moiety resulting in metronidazole's retention in the GI tract. This permitted us to evaluate how drug absorption affects treatment outcomes with metronidazole. This study suggests that even if metronidazole was non-absorbed that treatment outcomes may still be poorer than vancomycin. However, we must emphasize that these results were obtained in the hamster model of acute CDI that responds exceptionally well to vancomycin, but also has limitations.[36] Unlike humans and mice, hamsters are uniquely susceptible to CDI and other GI diseases.[36] Recently, Warren *et al.*[37] reported that in the murine model of CDI, metronidazole was superior to vancomycin and fidaxomicin, where metronidazole-treated animals failed to show relapse following treatment. This only serves to highlight current challenges in animal models for CDI.[36] We speculate that minimally absorbed derivatives of metronidazole, such as those reported herein, are also likely to demonstrate better efficacy in other CDI clinical settings. However, like metronidazole, the metronidazole–tetramic acid compounds were active against key gut flora, including *Bacteroides* species that contribute to preventing recurrence as well as maintaining the homeostasis of the immune system.[38] This is not desirable for new anti-*C. difficile* agents and the development of future agents needs to take this into account.

In our study, the tetramic acid motif did not contribute to the inhibitory function of the hybrids, presumably as they did not have the requisite charge distribution of reutericyclin to be membrane active. This result was surprising given that this pharmacophore is found in several narrow-spectrum antimicrobial natural products and the propensity of the tetramic acid motif to interact with different catalytic or allosteric sites of essential bacterial enzymes.[7,9,39] Therefore, in spite of containing the 3-acyltetramic acid, which has several reported modes of action from protein target inhibition to dissipation of the bacterial membrane potential,[7] the antimicrobial activity of compounds in the hybrid series appears to be solely dependent on the nitro group from metronidazole. Thus, the main contribution of the tetramic acid was in restricting the absorption of the hybrids from the GI tract, through an unknown mechanism. This idea of deploying the tetramic acid core to retain drugs in the GI tract or at least lowering absorption is a novel finding, which could lead to this core being utilized for delivering drugs intended as treatments for gastrointestine-specific microbial infections or colon cancer. Importantly, this approach may prove significant in lowering

side effects resulting from systemic circulation of drugs, such as metronidazole and other nitroheterocyclic drugs.

## Acknowledgements

We thank Drs Mark Wilcox and Jane Freeman at the University of Leeds, UK for providing metronidazole-resistant clinical strains of *C. difficile*. We thank Yizhe Chen from Department of Chemical Biology and Therapeutics, SJCRH, for analysing the pharmacokinetic data.

## Funding

This work was funded by Grant 5R01AT006732 from the National Center for Complementary and Alternative Medicine at the National Institutes of Health, with additional funds from the American Lebanese Syrian Associated Charities (ALSAC), St Jude Children's Research Hospital.

## Transparency declarations

None to declare.

## Supplementary data

Further experimental details, Figures S1 to S4 and Tables S1 to S12 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

## References

**1** Tsutsumi LS, Owusu YB, Hurdle JG *et al*. Progress in the discovery of treatments for *C. difficile* infection: a clinical and medicinal chemistry review. *Curr Top Med Chem* 2014; **14**: 152–75.

**2** Cohen SH, Gerding DN, Johnson S *et al*. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect Control Hosp Epidemiol* 2010; **31**: 431–55.

**3** Pepin J. Vancomycin for the treatment of *Clostridium difficile* infection: for whom is this expensive bullet really magic? *Clin Infect Dis* 2008; **46**: 1493–8.

**4** Johnson S, Louie TJ, Gerding DN *et al*. Vancomycin, metronidazole, or tolevamer for *Clostridium difficile* infection: results from two multinational, randomized, controlled trials. *Clin Infect Dis* 2014; **59**: 345–54.

**5** Wilcox MH. Editorial Commentary: The trials and tribulations of treating *Clostridium difficile* infection—one step backward, one step forward, but still progress. *Clin Infect Dis* 2014; **59**: 355–7.

**6** Hurdle JG, Heathcott A, Yang L *et al*. Reutericyclin and related analogues kill stationary phase *Clostridium difficile* at achievable colonic concentrations. *J Antimicrob Chemother* 2011; **66**: 1773–6.

**7** Schobert R, Schlenk A. Tetramic and tetranic acids: an update on new derivatives and biological aspects. *Biorg Med Chem* 2008; **16**: 4203–21.

**8** Zhu W, Zhang Y, Sinko W *et al*. Antibacterial drug leads targeting isoprenoid biosynthesis. *Proc Natl Acad Sci USA* 2013; **110**: 123–8.

**9** Lu J, Patel S, Sharma N *et al*. Structures of kibdelomycin bound to *Staphylococcus aureus* GyrB and ParE showed a novel U-shaped binding mode. *ACS Chem Biol* 2014; **9**: 2023–31.

**10** Pronin SV, Martinez A, Kuznedelov K *et al*. Chemical synthesis enables biochemical and antibacterial evaluation of streptolydigin antibiotics. *J Am Chem Soc* 2011; **133**: 12172–84.

**11** Cherian PT, Wu X, Maddox MM *et al*. Chemical modulation of the biological activity of reutericyclin: a membrane-active antibiotic from *Lactobacillus reuteri*. *Sci Rep* 2014; **4**: 4721.

**12** Wu X, Cherian PT, Lee RE *et al*. The membrane as a target for controlling hypervirulent *Clostridium difficile* infections. *J Antimicrob Chemother* 2013; **68**: 806–15.

**13** Ueda C, Tateda K, Horikawa M *et al*. Anti-*Clostridium difficile* potential of tetramic acid derivatives from *Pseudomonas aeruginosa* quorum-sensing autoinducers. *Antimicrob Agents Chemother* 2010; **54**: 683–8.

**14** Fukuyama T, Jow C-K, Cheung M. 2- and 4-Nitrobenzenesulfonamides: exceptionally versatile means for preparation of secondary amines and protection of amines. *Tetrahedron Lett* 1995; **36**: 6373–4.

**15** Narayan RS, Vannieuwenhze MS. Versatile and stereoselective syntheses of orthogonally protected β-methylcysteine and β-methyllanthionine. *Org Lett* 2005; **7**: 2655–8.

**16** Lacey RN. Derivatives of acetoacetic acid. Part IV. A new route to α-acetyltetronic acids. *J Chem Soc* 1954: 832–9.

**17** Lacey RN. Derivatives of acetoacetic acid. Part VII. α-Acetyltetramic acids. *J Chem Soc* 1954; 850–4.

**18** Bertinaria M, Galli U, Sorba G *et al*. Synthesis and anti-*Helicobacter pylori* properties of NO-donor/metronidazole hybrids and related compounds. *Drug Dev Res* 2003; **60**: 225–39.

**19** Peukert YS, Zhang R, Hurley B *et al*. Design and structure–activity relationships of potent and selective inhibitors of undecaprenyl pyrophosphate synthase (UPPS): tetramic, tetronic acids and dihydropyridin-2-ones. *Biorg Med Chem Lett* 2008; **18**: 1840–4.

**20** Mirzaei J, Pirelahi H, Amini M *et al*. Convenient syntheses of 5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-1,3,4-oxadiazole-2(3H)thione and N-substituted 2-amino-5-[(2-methyl-5-nitro-1H-imidazol-1-yl)methyl]-1,3,4-thiadiazoles. *J Heterocyclic Chem* 2008; **45**: 921.

**21** Schobert R, Dietrich M, Mullen G *et al*. Phosphorus ylide based functionalizations of tetronic and tetramic acids. *Synthesis* 2006; **22**: 3902–14.

**22** Jeong YC, Moloney MG. Synthesis of and tautomerism in 3-acyltetramic acids. *J Org Chem* 2011; **76**: 1342–54.

**23** Wu X, Alam MZ, Feng L *et al*. Prospects for flavonoid and related phytochemicals as nature-inspired treatments for *Clostridium difficile* infection. *J Appl Microb* 2014; **116**: 23–31.

**24** Zhai S, Yang L, Cui QC *et al*. Tumor cellular proteasome inhibition and growth suppression by 8-hydroxyquinoline and clioquinol requires their capabilities to bind copper and transport copper into cells. *J Biol Inorg Chem* 2010; **15**: 259–69.

**25** Anton PM, O'Brien M, Kokkotou E *et al*. Rifalazil treats and prevents relapse of *Clostridium difficile*-associated diarrhea in hamsters. *Antimicrob Agents Chemother* 2004; **48**: 3975–9.

**26** Kumar M, Adhikari S, Hurdle JG. Action of nitroheterocyclic drugs against *Clostridium difficile*. *Int J Antimicrob Agents* 2014; **44**: 314–9.

**27** Saadeh HA, Mosleh IM, Mubarak MS. Synthesis of novel hybrid molecules from precursors with known antiparasitic activity. *Molecules* 2009; **14**: 1483–94.

**28** Charmot D. Non-systemic drugs: a critical review. *Curr Pharm Des* 2012; **18**: 1434–45.

**29** Castillo-Garit JA, Marrero-Ponce Y, Torrens F *et al*. Estimation of ADME properties in drug discovery: predicting Caco-2 cell permeability using atom-based stochastic and non-stochastic linear indices. *J Pharm Sci* 2008; **97**: 1946–76.

**30** Goulding D, Thompson H, Emerson J *et al*. Distinctive profiles of infection and pathology in hamsters infected with *Clostridium difficile* strains 630 and B1. *Infect Immun* 2009; **77**: 5478–85.

**31** Moreno SNJ, Decampo R. Mechanism of toxicity of nitro compounds used in the chemotherapy of trichomoniasis. *Environ Health Perspect* 1985; **64**: 199–208.

**32** Muller M. Reductive activation of nitroimidazoles in anaerobic microorganisms. *Biochem Pharmacol* 1986; **35**: 37–41.

**33** Miyamoto Y, Kalisiak J, Korthals K *et al*. Expanded therapeutic potential in activity space of next-generation 5-nitroimidazole antimicrobials with broad structural diversity. *Proc Natl Acad Sci USA* 2013; **110**: 17564–9.

**34** Kim D, Hong S, Jung S *et al*. Synthesis and evaluation of N-nicotinoyl-2-{2-(2-methyl-5-nitroimidazol-1-yl)ethyloxy}-D,L-glycine as a colon-specific prodrug of metronidazole. *J Pharm Sci* 2009; **98**: 4161–9.

**35** Lofmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis* 2010; **50** Suppl 1: S16–23.

**36** Hutton ML, Mackin KE, Chakravorty A *et al*. Small animal models for the study of *Clostridium difficile* disease pathogenesis. *FEMS Microbiol Lett* 2014; **352**: 140–9.

**37** Warren CA, van Opstal EJ, Riggins MS *et al*. Vancomycin treatment's association with delayed intestinal tissue injury, clostridial overgrowth, and recurrence of *Clostridium difficile* infection in mice. *Antimicrob Agents Chemother* 2013; **57**: 689–96.

**38** Ubeda C, Pamer EG. Antibiotics, microbiota, and immune defense. *Trends Immunol* 2010; **33**: 459–66.

**39** Lee LV, Granda B, Dean K *et al*. Biophysical investigation of the mode of inhibition of tetramic acids, the allosteric inhibitors of undecaprenyl pyrophosphate synthase. *Biochemistry* 2010; **49**: 5366–76.