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

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‡These authors conceived, designed and supervised the study.

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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 gastrointestinal-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, which is mediated by the production of toxins TcdA and TcdB, which are responsible for tissue inflammation and epithelial damage. The antibiotics metronidazole and vancomycin are the current first-line treatments for mild and severe forms of CDI, respectively. 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. However, this treatment paradigm has changed in the setting of severe CDI, 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. 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.3,4 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 moiety6 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 antibiotic 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 DNA 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 acetylated with a ketene–acetonitrile 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 conditions18 while the 3-methoxycarbonyl tetramic acid was synthesized from Leu-OH and methyl malonyl chloride using Lacey–Dieckmann conditions. Reaction of these two intermediates in a microwave19 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 oxidation20 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 13C- and 1H-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 previously22 in brain heart infusion (BHI) broth in 96-well microtiter plates, with a bacterial inoculum of ≏106 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 (≏107 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 106 cfu/mL of the C. difficile strain ATCC 43596 that was grown in sporation medium and washed once with pre-reduced PBS,13 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 ≥ 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 microtubes containing caecal material (~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. 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 might 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 (Papp, 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
Table 1. Design and activities of metronidazole–tetramic acid hybrids

<table>
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<th>MIC (mg/L)</th>
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<th>BAA-1875</th>
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</table>

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—inobutyl, 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 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

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Continued
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).

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 [$C_{\text{max}}$ (mg/L): metronidazole, 51.04; 2344, 2.11 x 10^{-1}; 2345, 6.16 x 10^{-1} and total exposure AUC (mg.h/L): metronidazole 135.29; 2344, 3.86 x 10^{-1}; 2345, 3.55 x 10^{-1}; Table S4]. The $T_{\text{max}}$ 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.
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 $1.0 \times 10^{-2}$ 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, 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 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 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, 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 for de novo resistance (Table S11).
Table 4. Comparison of mechanism of action of nitro and des-nitro metronidazole and analogues

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<th>Compound</th>
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</table>

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. Nitroheterocyclic prodrugs continue to represent attractive treatment approaches for anaerobic GI and systemic infections caused by protozoa and bacterial pathogens. However, metronidazole and other members within this drug class are not typically developed to be retained in the GI tract for localized treatment. Recent clinical findings by Johnson et al. 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. Unlike humans and mice, hamsters are uniquely susceptible to CDI and other GI diseases. Recently, Warren et al. 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. 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. 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. 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, 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 gastrointestinal-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.

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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/).

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