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Action of nitroheterocyclic drugs against *Clostridium difficile*

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ABSTRACT

The nitroheterocyclic classes of drugs have a long history of use in treating anaerobic infections, as exemplified by metronidazole as a first-line treatment for mild-to-moderate *Clostridium difficile* infection (CDI). Since direct comparisons of the three major classes of nitroheterocyclic drugs (i.e. nitroimidazole, nitazoxanide and nitrofurans) and nitrosating agents against *C. difficile* are under-examined, in this study their actions against *C. difficile* were compared. Results show that whilst transient resistance occurs to metronidazole and nitazoxanide, stable resistance arises to nitrofurans upon serial passage. All compounds killed *C. difficile* at high concentrations in addition to the host defence nitrosating agent S-nitrosoglutathione (GSNO). This suggests that GSNO killing of *C. difficile* contributes to its efficacy in murine CDI. Although nitric oxide production could not be detected for the nitroheterocyclic drugs, the cellular response to metronidazole and nitrofurans has some overlap with the response to GSNO, causing significant upregulation of the hybrid-cluster protein Hcp that responds to nitrosative stress. These findings provide new insights into the action of nitroheterocyclic drugs against *C. difficile*.

1. Introduction

Clostridium difficile is the main cause of hospital-acquired diarrhoea in developed countries such as the USA and Europe. Each year in the USA alone, there are >400,000 cases of *C. difficile* infection (CDI), with >14,000 deaths [1]. Since the 1980s, metronidazole, a 5-nitroimidazole prodrug, has been established as a first-line therapy for mild-to-moderate CDI [2]. Despite its long history of use for treating CDI, the cellular action of metronidazole against *C. difficile* is not well characterised [3]. However, based on studies in other organisms, metronidazole is bioreductively activated by cellular oxidoreductases (e.g. nitroreductases), whereby its nitro group is reduced by an electron to produce a highly reactive and unstable nitroimidazole anion that can have several fates [4]. The unstable nitroimidazole anion may be further reduced to nitroso and hydroxylamine intermediates or may undergo decomposition yielding additional reactive species in the form of an imidazole radical and a nitrite anion from which nitric oxide (NO) is derived [4]. These nitroimidazole reactive derivatives and NO cause damage to cellular targets, namely proteins and DNA, leading to cell death [4,5]. If NO is produced upon metronidazole bioreduction in *C. difficile*, this might indicate that it imposes nitrosative stress in *C. difficile* in a manner similar to the innate immune system [6]. However, the genetic response of *C. difficile* both to nitroheterocyclic drugs, including metronidazole, and host-derived NO-generating molecules such as S-nitrosoglutathione (GSNO) is relatively under-characterised [7].

Interestingly, there are only a few reports of metronidazole resistance in *C. difficile* [3,8]. This extremely low incidence of metronidazole resistance in *C. difficile* is confounded by the instability of the metronidazole-resistant phenotype, with resistance being lost during freezer storage or following brief passage in microbiological media [3,9]. The rarity of metronidazole resistance in *C. difficile* is unusual considering that resistance to metronidazole occurs by several different mechanisms in other bacteria [10]. This prompted us to question whether the lack of metronidazole resistance in *C. difficile* is also displayed by other nitroheterocyclic drugs. Besides metronidazole, other members of the nitroheterocyclic drug class are also important treatments for other anaerobic infections, namely the 5-nitrofuran and nitrothiazolyl drugs [11,12].

Furthermore, the nitrothiazolyl nitazoxanide is considered an alternative treatment for CDI and has been successfully modified to produce improved analogues [12]. A key difference in these three nitroheterocyclic drug types arises from their redox potential, which dictates the spectrum of activity, mechanism of bioreduction and cellular effects [13]. Interestingly, nitazoxanide acts as a non-competitive inhibitor of pyruvate:ferredoxin oxidoreductase (PFOR) in anaerobes (e.g. *C. difficile*), protozoa and *Helicobacter pylori*, which is distinct to the cellular action of 5-nitroimidazoles and 5-nitrofurans, involving the formation of reactive species and inhibition of multiple targets [10].

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In this study, we sought to better understand the action of these three subclasses of nitroheterocyclic drugs against *C. difficile* by directly comparing their effects on cell viability, propensity to select for stable resistance, and the cellular responses of DNA damage and nitrosative stress. The results suggest that all three nitroheterocyclic subclasses show characteristic mode of action profiles, with nitroimidazoles and nitrofurans bearing some resemblance to GSNO. We also report for the first time that *C. difficile* is rapidly killed by GSNO, which now provides an additional basis for the observed efficacy of this molecule in mice with CDI [6].

2. Materials and methods

2.1. Chemicals, bacterial strains and growth conditions

Clostridium difficile strains CD196 (a historic NAP1 strain) and R20291 (a contemporary NAP1 strain) were kindly provided by Dr A.L. Sonenshein (Tufts University, Medford, MA) and strain BAA-1875 (NAP7) was obtained from ATCC (Manassas, VA). All strains were routinely grown in pre-reduced BHITY broth [brain–heart infusion, tryptone (1%, w/v) and yeast extract (0.5%, w/v)] at 37 °C in a Whitley A35 anaerobic workstation (Don Whitley Scientific, Shipley, UK). Antibiotics were obtained from the following sources: metronidazole, vancomycin, fusidic acid, rifaximin and nitazoxanide were from Sigma–Aldrich (St Louis, MO); ornidazole was from Alfa Aesar (Ward Hill, MA); nitrofurazone was from TCI America (Portland, OR); nifuroxazide and furazolidone were from MP Biomedicals (Santa Ana, CA); and nitrofurantoin was from Acros Organics (Fair Lawn, NJ). GSNO was from Enzo Life Sciences (Farmingdale, NY) and was prepared fresh in culture medium adjusted to pH 6.5 and was tested in medium of pH 6.5. Stock solutions of other compounds were stored at −80 °C in dimethyl sulphoxide (DMSO) at 10 mg/mL.

2.2. Determination of minimum inhibitory concentrations (MICs)

MICs of compounds were determined in 24-well plates, or in 96-well round-bottom microtitre plates (Thermo Scientific, Waltham, MA) for freeze–thawed cultures from serial passage experiments [14]. In 24-well plates, compounds were first two-fold serially diluted in 500 μL of BHITY broth, followed by the addition of 500 μL of 10^6 CFU/mL of *C. difficile*, with test concentrations ranging from 0.06 to 64 μg/mL. Plates were incubated anaerobically at 37 °C for 24 h. MICs were similarly determined in 96-well microtitre plates but using antibiotic dilution volumes of 50 μL with a final volume of 100 μL after addition of the inoculum. There was no difference in the MICs determined in 96-well microtitre plates or 24-well plates.

2.3. Determination of minimum bactericidal concentrations (MBCs)

MBCs were determined against log-phase cells in 24-well plates as described previously [14]. Briefly, 900 μL of mid-logarithmic cells [optical density at 600 nm (OD_{600}) ≈ 0.3] was added to 100 μL of two-fold serially diluted compounds. After 24 h of incubation, viable counts were enumerated on BHITY agar containing 20% (w/v) activated charcoal [14]. MBCs were determined at least twice and were defined as the lowest concentration of compound killing ≥3 log of the initial inoculum (ca. 10^7 CFU/mL).

2.4. Time–kill assay

Time–kill assay experiments were performed as described previously [14]. Mid-logarithmic phase cultures (OD_{600} ≈ 0.3) were exposed to metronidazole and nitazoxanide at their MBC, nitrofurantoin at 8× and 16× its MIC and S-nitrosoglutathione (GSNO) at its MIC. Total viable counts were performed on samples recovered at different points. All experiments were performed at least twice.

2.5. Selection of nitroheterocyclic-resistant mutants

To compare the potential for emergence of de novo resistance in *C. difficile*, methods for plating of concentrated cultures or serial passage in the presence of subinhibitory concentrations of drug were adopted as described below.

2.5.1. Mutant selection on agar

Overnight cultures in BHITY broth (10 mL) were concentrated 10-fold, resulting in 10^9 CFU/mL. The entire 1 mL of sample was plated (100 μL per plate) onto BHITY agars containing antibiotic at 4× MIC. Following incubation for 48 h, the mutation frequency was obtained from the total number of colonies on selection plates divided by the total number of viable cells.

2.5.2. Selection of stable resistance by serial passage

Serial passage experiments were conducted in 96-DeepWell plates (Thermo Scientific), essentially as described previously [15] except for the use of BHITY broth (1 mL) as the growth medium. Initially, compounds were two-fold serially diluted to yield antibiotic concentrations that were eight-fold above and four-fold below their MICs. Each well was inoculated with *C. difficile* to yield ca. 10^6 CFU/well. Following incubation for 24 h at 37 °C, the lowest concentration of compound preventing visible growth was recorded as the MIC. Bacteria growing one dilution below the MIC were then used to inoculate the subsequent round of passaging in fresh broth containing antibiotic. This was repeated for a total of 20 passages and at each stage the MIC obtained was used to define the compound concentration ranges for the subsequent passage. To isolate individual colonies from populations showing growth at elevated concentrations, cultures were plated onto agar with antibiotic at 4× MIC. To determine whether stable resistance to compounds arose during passaging, stocks of bacteria were first stored at −80 °C. For every fourth passage in the series, frozen stocks were plated onto non-selective BHITY agar. After overnight growth, the entire plate was scrapped into fresh broth, incubated overnight and MICs were determined in 96-well microtitre plates.

2.6. Measurement of nitrite and nitric oxide

Two methods were used to detect the production of reactive nitrite or NO in cultures as described below.

2.6.1. Griess reagent assay

Since the initial experiments revealed quenching of Griess reagent assay in BHITY, these experiments were performed in TYG broth (1%, w/v tryptone, 0.5%, w/v yeast and 1%, w/v glucose). There were no differences in the MICs of compounds in TYG or BHITY broth. Aliquots (1 mL) of concentrated *C. difficile* CD196 (ca. 10^9 CFU/mL) were exposed to metronidazole and nitrofurantoin at 64 μg/mL (0.37 mM and 0.27 mM, respectively) and to GSNO (50.4 μg/mL; 0.15 mM) for 30 min and 90 min. Cells were centrifuged and supernatants were retained, whereas cell pellets were lysed by bead beating. Both the supernatant and cell lysates (50 μL) were assayed for nitrite according to instructions for the Griess reagent kit from Promega (Madison, WI). Nitrite concentrations were determined from a standard curve of nitrite at 1.5–200 μM; the limit of detection was 2.5 μM.

2.6.2. β-Galactosidase (β-gal) reporter assay

Escherichia coli strain JOEY426 (MG1655 derivative of JOEY19 [16]) carrying a NO-inducible promoter (*ytfE*) fused to *lacZ* was