Preclinical Studies of Amixicile: A Systemic Therapeutic Developed for Treatment of Clostridium difficile Infections also shows Efficacy against Helicobacter pylori

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Running Title: Amixicile preclinical evaluation

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Abstract

Amixicile shows efficacy in treatment of Clostridium difficile infections (CDI) in a mouse model with no recurrence of CDI. Since amixicile selectively inhibits the action of a B-vitamin (thiamine pyrophosphate) cofactor of pyruvate: ferredoxin oxidoreductase (PFOR), it may both escape mutation based drug resistance and spare beneficial probiotic gut bacteria that do not express this enzyme. Amixicile is a water soluble derivative of nitazoxanide, an antiparasitic therapeutic that also shows efficacy against CDI in humans. In comparative studies, amixicile showed no toxicity for hepatocytes at 200 μM (NTZ was toxic above 10 μM), was not metabolized by human, dog or rat liver microsomes, showed equivalence with NTZ or better in CYP assays, and did not activate efflux pumps (BCRP, P-gp). Amixicile was well tolerated by oral or intraperitoneal routes at maximum doses (300 mg/kg) in mice and rats. Plasma exposure (rats) based on AUC was 79.3 hr*μg/ml (30 mg/kg dose) to 328 hr*μg/ml (100 mg/kg dose), C<sub>max</sub> of 20 μg/ml, T<sub>max</sub> of 0.5 to 1 hr and a T<sub>1/2</sub> of 5.6 hr. Amixicile did not concentrate in mouse stools or adversely affect gut populations of Bacteroides sp, firmicutes, segmented filamentous bacteria or Lactobacillus sp. Systemic bioavailability was demonstrated through eradication of Helicobacter pylori in a mouse infection model. In summary, the efficacy of amixicile in treating CDI and other infections, together with low toxicity, an absence of mutation-based drug resistance and excellent DMPK metrics suggest potential for broad application in treatment of infections caused by PFOR-expressing microbial pathogens in addition to CDI.
INTRODUCTION

*Clostridium difficile*, a Gram-positive spore-forming obligate anaerobe present in the intestinal microflora of most humans and animals, is an important cause of antibiotic-associated infectious diarrhea and pseudomembranous colitis. *C. difficile* infection (CDI) is the leading cause of healthcare-associated infectious diarrhea that is attributed to emergence of hypervirulent, binary toxin-producing strains such as the North American pulsed-field type 1 (NAP1/BI/027) (1,2,3,4). Antibiotic interventions with oral vancomycin and metronidazole (MTZ) are effective treatments for severe and mild forms of the disease, respectively (5,6); but recurrence rates of 25% or higher are common and the risk of chronic CDI episodes increases to 60% (2). Whether recurrence is the result of eradication failure or to reinfection, it is generally believed that susceptibility to CDI is a function of the species diversity and type of resident gut microflora which serve as a protective barrier to colonization (7). Even standard CDI therapies with metronidazole, vancomycin and fidaxomicin impair intestinal flora and thereby contribute to continued susceptibility and recurrence of CDI (6,8,9). The importance of fecal microflora is underscored by the success rates of 80 – 90% achieved with fecal transplants from healthy volunteers (10) and with implementation of probiotic preventive measures that lower the incidence of recurrence by 66% (11). Among attributes believed important in development of newer therapeutics to treat CDI include emphasis on selectivity for *C. difficile* and on retention of the drug within the intestine.

We initially developed amixicile, a bioavailable derivative of nitazoxanide (NTZ), to treat systemic infections caused by strictly anaerobic bacteria, anaerobic parasites and gastrointestinal infections caused by *Helicobacter pylori* and *Campylobacter jejuni*, all of which express pyruvate: ferredoxin oxidoreductase (PFOR) and related enzymes (12-15). NTZ shows good *in vitro* efficacy against these microorganisms, but is limited clinically to treatment of intestinal infections caused by *Cryptosporidium parvum* and *Giardia lamblia* (16). However, since NTZ was found to be noninferior to MTZ in treatment of CDI in a randomized double-blind...
prospective patient trial (8,17), we evaluated the efficacy of amxicile in a mouse CDI model (15). In this model, infected mice develop diarrhea, lose weight, and succumb on days 2 to 6 after oral inoculation with $10^4$ to $10^5$ colony forming units (cfu) of *C. difficile* (6,15). Amxicile proved superior to NTZ in this model. In an optimized CDI mouse model, amxicile showed equivalence with vancomycin and fidaxomicin at day 5 and superiority by day 12 (15). Recurrence was common in mice treated with vancomycin or fidaxomicin, whereas no recurrence was observed in mice receiving amxicile (6,15). In fact, in all of our studies with mice treated with NTZ or tested analogues of NTZ, none of the surviving animals relapsed (6,15). We concluded that gut repopulation with beneficial (non-PFOR) bacteria, considered essential for protection against CDI, rebounds much sooner with amxicile therapy than with vancomycin or fidaxomicin (15). McVay and Rolfe reported that NTZ was active against CDI in a hamster model and noted that unlike vancomycin and MTZ, pretreatment of hamsters with NTZ did not induce CDI, which suggested that NTZ did not suppress protective resident flora (18). This conclusion is supported by several MIC based comparative studies showing that NTZ is not inhibitory to various species of *Lactobacillus*, *Propionibacteria* and *Bifidobacteria* which lack the PFOR target (19,20). In contrast, many of these bacteria are susceptible to fidaxomicin and vancomycin (21). Several studies found members of the genus *Bifidobacterium* to be increased in relative abundance in stools from individuals considered resistant to CDI infection compared with those susceptible to recurrence (22, 23). Many of these human microbiome studies of CDI and controls reveal substantial changes to a complex gut microflora, but have yet to correlate these changes with specific antibiotic therapy.

Here we report results of preclinical studies which showed that amxicile was not cytotoxic or metabolized by liver microsome fractions and both dose range findings and PK studies in rats indicated that amxicile was safe, well tolerated, and achieved serum levels well in excess of the MIC for *C. difficile*. Therapeutic bioavailability was further demonstrated by successful eradication of *H. pylori* infection in a mouse model. Our studies challenge
conventional wisdom that a pathogen specific therapeutic that concentrates in the gut is the most effective strategy for developing new CDI therapeutics. In contrast, we suggest that a systemic therapeutic like amixicile, by concentrating in areas of mucosal inflammation caused by *C. difficile* or *H. pylori*, would act locally and spare susceptible flora that are not associated with disease. Such therapeutics might prove beneficial when administered together with probiotic or fecal transplant treatments.

**Materials and Methods**

**Scale up of amixicile.** Scale up synthesis of amixicile related derivatives have been previously described (13,14). The chemical structures of amixicile and other analogues used in this study are depicted in Figure 1. Nitazoxanide was purchased commercially from Waterstone Technology (Carmel, IN). The purity of all compounds used in these studies was assessed by spectrophotometric, NMR and MS protocols (12,14,15).

**Bacterial strains.** *Helicobacter pylori* strains were grown on Brucella based medium supplemented with 7.5% new born serum and grown in a microaerobic environment as previously described (12). MIC was determined by microdilution for *H. pylori* strains SS1 and 26695 in Brain Heart Infusion broth (14, 15).

**Metabolic stability.** Metabolic stability was determined at 1 and 10 μM final concentration of compounds incubated with pooled human, rat and dog liver microsomes (0.5 mg protein/ ml) containing appropriate cofactors (2.5 mM NADPH and 3.3 mM MgCl₂) in 0.1 M phosphate buffer, pH 7.4, in a 37°C water bath. The incubation contained a final organic solvent concentration of 0.1% DMSO. Reactions were started with addition of microsomes and stopped by removing 100 μl aliquots at selected times (0, 15, 30 and 60 min) and mixing with 200 μl of acetonitrile containing an internal standard. Samples were transferred to a 96-well plate for further dilution, followed by LC-MS/MS analysis. Controls for metabolism included 10 μM
midazolam, a known substrate of CYP3A4 and incubation of test compounds and midazolam with heat inactivated microsomes for 0 and 60 min, as a negative control. All samples were assayed in triplicate.

**In vitro CYP inhibition.** Pooled human liver microsomes (0.5 mg/ml) and cofactors (2.5 mM NADPH and 3.3 mM MgCl₂) were incubated with test compounds (1 and 10 μM) and a cocktail of seven different CYP probe substrates in 0.1 M phosphate buffer, pH 7.4 (final volume of 200 μl). Substrates utilized included: 25 μM phenacetin (CYP1A2), 25 μM bupropion (CYP2B6), 10 μM diclofenac (CYP2C9), 20 μM mephenytoin (CYP2C19), 10 μM bufuralol (CYP2D6), 50 μM testosterone (CYP3A4) and 4 μM midazolam (CYP3A4). Specific inhibitor control samples were incubated and analyzed in the same manner as test compounds, but contained the following inhibitors in place of test article: 10 μM furafylline (CYP1A2), 10 μM TEPA (CYP2B6), 3 μM sulfaphenazole (CYP2C9), 10 μM nootkatone (CYP2C19), 2 μM quinidine (CYP2D6) and 5 μM ketoconazole (CYP3A4). Reactions were started with addition of microsomes and terminated after 20 min of incubation at 37°C by the addition of 200 ml of ice cold acetonitrile containing 2 μM dextrorphan (internal standard). Samples were centrifuged at 1500 rpm for 20 min at 10°C and supernatants collected and analyzed by LC-MS/MS by positive ion electrospray. The percent CYP activity in test articles or specific inhibitor samples relative to the control samples not containing test article or controls was calculated as follows: \[ \frac{\text{Substrate metabolite response (peak area ratio, PAR) in presence of inhibitor or test compound/substrate metabolite mean PAR in control}}{\times 100} \]. CYP enzyme activity in the presence of test compound that was less than 70% of control activity was considered significant inhibition in this assay.

**In vitro bi-directional permeability using Caco-2 cells.** CacoReady™ HTS Transwell®-24 plates consisting of differentiated Caco-2 cells plated on polycarbonate microporous filters (6.5 mm diameter, 0.33 cm² growth area, and 0.4 mm pore size) were obtained from ADMEcell, Inc. (Emeryville, CA). The cells were prepared for assay according to the manufacturer's
instructions and transport medium was replaced with DMEM (Dulbecco’s Modified Eagle Medium), and incubated for 72 h prior to incubation with test compounds. The integrity of the monolayer was assessed by measuring the transepithelial electrical resistance (TEER) using an epithelial volt ohm meter (EVOM Instrument, World Precision Instruments; Sarasota, FL). A TEER value >1000 Ωcm² indicated that the barrier system was acceptable. For apical-to-basal permeability (A-B), test and control compound solutions were prepared in HBSS (Hanks’ balanced salt solution) at pH 6.0 or 7.4 and added to the apical side of the cell monolayer. For basal-to-apical permeability (B-A), test and control compound solutions were prepared in HBSS at pH 7.4 and added to the basal side of the cell monolayer. Permeability was measured by aliquots from the receiving compartment. Samples in acetonitrile were stored at -80°C and analyzed by LC-MS/MS in multiple reaction monitoring mode using positive or negative-ion electrospray ionization. All assays were in triplicate and results presented as mean and SD.

Plasma protein binding studies. Plasma and PBS calibration standards were prepared in blank human, rat or dog plasma and PBS. Spiking solutions were prepared by diluting the test article, amixicile (10 mg/ml) or VPC16a1011 (5 mg/ml) DMSO stocks to give spiking standards at 3, 5, 10, 30, 100, 300, 500, 800, 1000, 1200 and 1500 μg/ml in DMSO. Five microliters of each spiking standard was spiked into 495 μl plasma (or PBS) to give calibration standards at 0.03, 0.05, 0.1, 0.3, 1, 3, 5, 8, 10, 12 and 15 μg/ml. The LLOQ for amixicile analysis was 0.05 μg/ml. Amixicile plasma samples for the dialysis experiments were prepared using the spiking solutions and 10 μl of each spiking solution (0.1, 1.0, or 10 mg/ml) were added to 990 μl plasma (human, dog and rat) to give samples at 1, 10, and 100 μg/ml. The samples were dialyzed against PBS at pH 7.4 using a Thermo Scientific Rapid Equilibrium Dialysis (RED) plate system with a cutoff of 8,000 Daltons. The chambers were incubated at 37°C for 4 hours. Upon completion, 50 μl of dialyzed plasma was diluted with an equal volume of PBS prior to protein precipitation. As a control for nonspecific binding to the filter, amixicile was tested in PBS in the
absence of serum. Samples were prepared for quantification of amixicile in dialysis samples (100 μl) to which 300 μl acetonitrile containing 10 μg/ml methyl nicotinate was added to precipitate plasma proteins. The supernatants following centrifugation were diluted in formic acid and analyzed by LC-MS/MS. A calibration standard curve was used to determine concentration of amixicile in each chamber and compared as Buffer chamber/Sample chamber. The percent amixicile bound to plasma protein was determined as the %Bound = 100 x amixicile in buffer chamber/mean values F equilibrium/amixicile in sample chamber and the mean and standard deviation were calculated using Microsoft Excel software.

**Induced mutation.** *E. coli* tester strain CC103 [araΔ(lac proB)/F' lacI lacZ proAB'] (24) was grown for 6 h in the presence of test compounds and then decimal dilutions were prepared in phosphate buffered saline (PBS) and spread-plated onto LB medium supplemented with 50 μg/ml of rifampin or onto LB medium without antibiotics (24). Two independent experiments, performed in triplicate, were averaged. The mutation frequency was determined as the number of rifampin resistant cfu/total cfu and presented as mutations per 10^8.

**Dose-range studies in Sprague Dawley (SD) rats.** Male SD rats (350 – 376 g) were obtained from Charles River (Hollister, CA). Animals were randomly distributed into groups of 3 based on weight and on day 1 were given the indicated dose of amixicile (20, 100, 200, or 300 mg/kg in 1% methyl cellulose) by oral gavage. Animals were monitored immediately and at 24 and 48 h and all surviving animals were euthanized after 48 h. General procedures for animal care and housing were in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals.*

**Plasma pharmacokinetic studies.** Jugular vein catheterized (JVC) SD rats were purchased from Charles River (300 – 350 g). Plasma drug levels of amixicile were determined in SD rats (3 per group) following a single oral dose of drug (30 or 100 mg/kg in 1% methyl cellulose in sterile water). Pre-dose and post-dose blood samples (50 μl) were collected at 5,
15, 30 min and 1, 2, 4, 8, 12 and 24 h (50 ml) by JVC and plasma was prepared within 30 min of collection. All plasma samples were stored at -70°C until analyzed. Calibration standards of amoxicilline were prepared in rat plasma from a 1 mg/ml stock solution in DMSO over a range of 0 to 2,500 ng/ml. Rat plasma protein (sample volume 50 μl) was precipitated with 200 μl of acetonitrile containing 1000 ng of VPC16b2031. VPC16b2031 is a dinitrothiophene derivative used as an internal standard (see Figure 1). The suspensions were clarified by centrifugation and 150 μl each was used for LC-MS/MS analysis. The calibration standard curve for amoxicilline was prepared by performing weighted 1/x2 quadratic regression of the peak area (PA) of amoxicilline as the dependent variable (y axis) and concentration as the independent variable (x axis).

\[
PA = a[\text{amoxicilline}]^2 + b[\text{amoxicilline}] + c
\]

The concentration of amoxicilline \( (x) = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \) was calculated using the Quanlynx portion of the Masslynx software. The goodness of fit of this standard curve is indicated by the coefficient of determination \( (r^2) \) obtained from the quadratic regression, with perfect fit yielding a \( r^2 \) of 1.000.

A non-compartmental model based on extravascular administration (oral gavage) was performed with WinNonlin (ver 5.2) using uniform weighting. The dose levels were entered as mg/kg so that no adjustment for body weight was needed. Data collected included the maximum plasma concentration \( (C_{\text{max}}) \), time at \( C_{\text{max}} \) (\( T_{\text{max}} \)), and the mean area under the plasma concentration time curve \( (AUC) \) up to the last measurable time point \( (AUC_{\text{last}}) \) and up to infinity \( (AUC_{\text{inf}}) \), terminal elimination half-life \( (t_{1/2}) \), apparent volume of distribution after oral administration \( (V/F) \) and total clearance after oral administration \( (Cl/F) \). Bioavailability was not determined since there was no iv dose used in this study. Terminal phase parameters \( (t_{1/2}, AUC_{\text{inf}}, Cl/F) \) were reported only when the goodness of fit \( (r^2) \) value of the best fit line in the terminal elimination phase was \( >0.85 \).
Pharmacokinetics was also performed on serum and stools from C57BL/6 male mice receiving a single oral dose of 200 mg/kg of amixicile. A spectrophotometric assay was developed based on the absorption of amixicile at 413 nm ($\varepsilon = 20 \text{ mM}^{-1}\text{cm}^{-1}$) (12, 15). Serum samples prepared from collected blood (15 min to 4 h and 24 h) were mixed with an equal volume of methanol to precipitate proteins. Following centrifugation, supernatants were diluted to 50% in PBS pH 7.4 and the concentration of amixicile was determined spectrophotometrically. A standard curve and limit of detection assays were determined by spiking mouse serum samples with a range of amixicile concentrations. The limit of detection in this assay was 0.1 $\mu$g/ml. Stool samples were suspended in PBS and similarly treated with equal volume of methanol. Supernatants were assayed for amixicile. The mean and standard deviation are reported on 3 samples each obtained from two mice per time point.

Animal studies. C57BL6/J mice were challenged with 3 doses of $5 \times 10^7$ CFU of *H. pylori* SS1 wild type strain in Brucella broth on days 1, 3, and 5 as previously described (25, 26). At two weeks post infection, groups of 5 mice each were treated by gavage with amixicile or metronidazole at 20 mg/kg as previously described (15, 27). For amixicile treated mice, a second group received two doses of 20 mg/kg each day. After 1 week post treatment, mice were sacrificed and stomachs were removed, homogenized and cfu was determined by plate count (25). Briefly, weighted gastric specimens were homogenized in phosphate-buffered saline (PBS) and plated in triplicate onto Columbia agar plates with selective antibiotics: vancomycin (10 $\mu$g/ml), trimethoprim (1 $\mu$g/ml), amphotericin B (5 $\mu$g/ml) and polymyxin B (5 $\mu$g/ml). Plates were incubated for 4 days at 37°C under microaerobic conditions (25, 27). Bacterial numbers are reported as mean and standard deviation of the number of CFU/g of stomach tissue. Statistical differences were determined using the Student t test, with $P$ values <0.05 considered significant.
Microbiota quantitative real-time RT-PCR: C57BL/6 mice (5-8 week old male and female) received 30 mg/kg (~600 μg/100 μl in PBS) of amixicile (8 mice) or PBS (7 mice) once daily for three days by oral gavage. The mice were sacrificed on day four and the intestine was collected for quantitative real time RTPCR (QPCR) analysis for selected microbial flora (28). Gene expression in terminal ileum was measured by real-time PCR utilizing Sybr Green and phyla and species level specific primers as previously described (28, 29). Data were normalized to a conserved *Eubacteria* 16s RNA gene (EUB). EUB primer sequences are as follows, EUB forward: 5'-ACTCCTACGGGAGGCAGCAGT-3', EUB reverse: 5'-ATTACCGCGGCTGCTGGC-3', SFB; SFB forward: 5'-GACGCTGAGGCATGAGAGCAT-3', SFB reverse: 5'-GACGGCACGGATTGTATTCA-3'. *Lactobacillus* sp. F: 5'-AGCAGTAG-GGAATCTTCCA-3', *Lactobacillus* sp. R: 5'-CACCGCTACACATGGAG-3'. *Bacteroides* F: 5'-GGTTCTGAGAGGAGGTCCC, *Bacteroides* R: 5'-GCTGCCTCCCGTAGGAGT-3'. *Firmicutes* F: 5'-GGAGYATGTGGTTTAATTCGAAGCA-3', *Firmicutes* R: 5'-AGCTGACGACAACCATGCAC-3'. The 16S rRNA results were normalized to the total bacteria.

RESULTS

Drug metabolism. The results of metabolic stability for amixicile incubated with human, rat and dog liver microsome fractions are presented in Table 1. After 1h, the remaining amixicile (10 μM) was 99 ± 5.2% for human, 96.5 ± 1.8% for rat, and 97.7 ± 2.4% for dog liver microsomes. These results were comparable with heat inactivated microsome controls indicating that amixicile is not appreciably metabolized by liver microsome fractions. In this assay, nitazoxanide (NTZ) was deacetylated to the phenol (tizoxanide) as previously reported (16, 30, 31). In CYP inhibition assays (data not presented), amixicile showed equivalence or lower inhibitory activities when compared with NTZ for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The potential for induction of CYPs was determined by QPCR over a concentration...
range from 0.6 to 200 μM for amixicile, but was limited to 10 μM for NTZ, due to toxicity. At 10 μM, neither NTZ nor amixicile showed any potential to activate CYPs. Amixicile was tested at higher concentrations and at the highest (200 μM) a wide variation was observed among the three sources of hepatocytes for CYP1A2 and CYP3A4, as one source was consistently high (up to 102-fold versus 10-fold for the two other sources). It is noteworthy that the absence of appreciable metabolism of amixicile by liver microsome fractions is consistent with previous studies showing that the 5-nitro group of amixicile and NTZ is not susceptible to nitroreduction (15, 32). These findings support previous conclusions indicating that NTZ and amixicile were not substrates of the NsfB nitroreductase of E. coli (15).

**Hepatocyte cytotoxicity.** Hepatocyte viability was measured by the MTT method and the cytotoxicity results are depicted in Table 2. Nitazoxanide was the most toxic with 99% of the monolayer destroyed at 25 μM (~8 μg/ml). NTZ was not toxic at 10 μM. Amixicile exhibited little cytotoxicity over a concentration range from 10 to 200 μM which was not considered significant since there was no dose dependent increase in toxicity. A second analogue tested for cytotoxicity was VPC16a1011 in which the benzene propylamine was replaced by a chloro-thiophene group (see Figure 1). VPC16a1011 was cytotoxic for hepatocytes at 5 μM, though in previous studies both VPC16a1011 and NTZ were shown to be less toxic for human foreskin cells (15).

**Mutation frequency.** We had previously shown that parent drug NTZ did not induce mutations in E. coli (32). To ensure that amixicile was not mutagenic, E. coli strain CC103 was exposed to 32 μg/ml of amixicile (~100 μM) for 6 h and then plated onto medium containing rifampicin (see Table 3). We also included NTZ and MTZ in the study. As a positive control, nitrofurazone was included as we had previously shown the drug to induce mutations in E. coli (24). As further control, we included VPC16b2031, which is a dinitrothiophene that showed weak activity in a nitroreductase assay. As seen in Table 3, the frequency of rifampin mutants
was not significant over controls for amixicile or NTZ. In this assay, both VPC16b2031 and MTZ showed an elevation in mutation frequency (ca 3 and 7 fold, respectively) and nitrofurazone increased mutation frequency by ~ 20 fold. Neither amixicile nor NTZ are substrates of nitroreductases and we conclude that these compounds are not mutagenic.

**Plasma binding studies.** Equilibrium dialysis was used to assess the extent of plasma binding by amixicile and VPC16a1011. As seen in Table 4, amixicile binding to plasma proteins was highest with human plasma (92% for 100 μg/ml) and somewhat less for dog and rat plasma. Relative aqueous solubility did not appear to be a factor as the cLogP for amixicile is 1.1 while for VPC16a1011 which binds plasma at >99% is insoluble in water (cLogP 3.0). The latter results are similar to that reported for NTZ (cLogP 2.2) which is 99% bound to plasma proteins (30, 31) and it is known that high protein concentrations affect potency of NTZ (MIC tests) for certain pathogens (33). We had previously reported that bovine serum albumin does not affect the MIC for amixicile (15).

**Absorption studies.** Bidirectional permeability was determined with established Caco-2 monolayers in transwell chambers. The ratio of $P_{app} \times 10^{-6}$ cm sec$^{-1}$ of apical to basal versus basal to apical was 3.6 at 1 μM and 3.3 at 10 μM consistent with the likelihood that amixicile is subject to efflux. This possibility was supported by inclusion of efflux inhibitor ketoconazole which increased uptake from apical to basal. However, we noticed that the pH on the apical side was acidic (pH 6.0) and the basal side was 7.4. Since the pK for amixicile is ca 6.2 (12), we considered the possibility that diffusion of the drug might be influenced by the anionic status. To test this possibility, the study was repeated with the apical pH set to 7.4 in HBSS. Under these conditions, the efflux ratio was 0.979. These studies also evaluated efflux via PCRP and P-gp and found efflux ratios below 2 indicating that amixicile is not a substrate for efflux in the Caco-2 model. Further studies showed that amixicile is not a substrate of OAT1, OAT3, OCT2, OATP1B1 or OATP1B3 (data not presented). Taken together, these studies suggest that
amxicile shows bidirectional permeability and is likely to be absorbed. These studies do raise
the possibility that uptake of amxicile may be sensitive to changes in local pH and to possible
differences in absorption between the anion and base.

**Dose range studies.** Previous dose range studies in mice had shown that amxicile was
well tolerated when administered by oral and intraperitoneal (i.p.) routes at 200 mg/kg (15). We
noted in previous studies that many of the NTZ analogues, including NTZ were lethal when
injected by the IP route (34). To further evaluate safety of amxicile, dose range studies were
performed in rats. Amxicile was well tolerated at the highest concentrations (300 mg/kg)
administered by oral gavage. The animals were observed for 48 h and there were no changes in
animal behavior or activity when compared to controls.

**Pharmacokinetics of amxicile in rats and mice.** Plasma pharmacokinetics were
determined in rats following a single dose of amxicile (30 or 100 mg/kg) by oral gavage (Figure
2A). Peak plasma levels (Tmax) were reached between 30 min and 1 h and amxicile was
quantifiable at 24 h post dose. Plasma levels for the 100 mg/kg dose remained high throughout
the study (Figure 2A open circles). The means for Cmax for both doses were 15.5 μg/ml and 19.7
μg/ml, respectively and were not statistically different (see Table 5). However, the AUC (area
under the curve) values for the two drug concentrations showed a three-fold difference (79.2
hr-μg/m versus 328 hr-μg/ml) as well as in T1/2 (1.5 hr versus 5.6 hr). These results may suggest
that absorption of amxicile decreases with drug concentration or elimination might be slowed by
saturation of clearance mechanisms. When similar plasma pharmacokinetics were determined
in mice receiving a single dose of 200 mg/kg in PBS, we noted that amxicile was more
efficiently absorbed with a Cmax of 179 μg/ml within 15 min and then achieved a steady level
between 40 and 60 μg/ml for 4 h (see Figure 2B). Since in the rat studies, amxicile was
suspended in 1% methylcellulose for the oral gavage, we believe that the 100 mg/kg drug dose in the rat likely limited absorption by creating a time-release profile that was not seen in the mouse study in the absence of carrier. In both studies, the high plasma levels are consistent with good absorption of amoxicillic and when the $C_{\text{max}}$/MIC is computed for the various susceptible pathogens, ratios above 150 would be predictive of clinical success. Consistent with good absorption metrics for amoxicillic, the presence of amoxicillic in stools of mice receiving 200 mg/kg was below detectable levels ($\sim 0.1 \mu g/ml$) when compared to a standard curve produced with amoxicillic-spiked stool samples. Routes of excretion were not investigated in this study.

**Therapeutic bioavailability.** To test whether plasma protein binding might affect the bioavailability and therefore the therapeutic efficacy of amoxicillic, we used a mouse model of *H. pylori* infection where plasma drug levels above MIC are required for eradication. Both human and animal studies have established that NTZ is not an effective therapy for *H. pylori* infections, despite excellent *in vitro* efficacy (15, 35). In contrast, metronidazole is an effective therapeutic for eradication of *H. pylori* infection in mice, but not as a monotherapy in humans or when strains are resistant to MTZ (27, 36). As seen in Figure 3, a single 20 mg/kg dose of amoxicillic was sufficient to decrease bacterial numbers (cfu/mg stomach tissue) by >2 logs in comparison with a similar dose of MTZ which decreased bacterial numbers by nearly 3 logs. Our studies showed that two 20 mg/kg doses of amoxicillic was nearly equivalent to the single dose of MTZ. These results show that plasma protein binding noted for amoxicillic does not appear to affect bioavailability or potency against *H. pylori* in the mouse model. Based on $C_{\text{max}}$ determined in mice receiving 200 mg/kg of amoxicillic (179 $\mu g/ml$), the estimated serum levels from a 20 mg/kg dose of amoxicillic ($>17 \mu g/ml$) would be in excess of the MIC for the SS1 strain of *H. pylori* ($<1.0 \mu g/ml$) and for *C. difficile* determined previously (15). We conclude that plasma protein binding does not affect bioavailability or therapeutic efficacy of amoxicillic.
Effect of amixicile on resident mouse flora. In vitro studies have shown that NTZ exhibits broad spectrum inhibitory activity against strictly anaerobic bacteria and our previous studies had shown that amixicile was particularly potent against Bacteroides fragilis in vitro (15). To evaluate the potential that amixicile might disrupt resident anaerobic flora, we randomized 15 mice of varying ages into two groups, one group receiving 30 mg/kg/day of amixicile and one receiving PBS for three days. On day four, the Bacteroides, segmented filamentous bacteria, firmicutes, and lactobacilli populations were analyzed by qPCR. As seen in Figure 4, there were no significant changes in these gut microflora populations over controls. These findings are consistent with our findings that amixicile does not accumulate in detectable concentrations in the stools of mice (< 0.1 μg/ml).

Discussion

Amixicile, a water soluble derivative of nitazoxanide (NTZ), selectively interferes with the biological function of thiamine pyrophosphate (TPP), the vitamin B1 cofactor of pyruvate:ferredoxin oxidoreductase (PFOR) and related α-ketoacid: ferredoxin oxidoreductases (12). These essential enzymes are ubiquitous to obligate anaerobic bacteria, human intestinal parasites, the archea and members of the epsilon proteobacteria (12,13). Other TPP containing enzymes, such as pyruvate dehydrogenase are not inhibited by amixicile. Conceptually, drugs that target the function of vitamins, themselves small molecules, are unlikely to undergo mutation to drug resistance without losing biological function. Such therapeutics might revolutionize treatment strategies for chronic infections that often require extended periods of antimicrobial intervention.

Amixicile demonstrated improved selectivity for the PFOR drug target and had lost many of the off target activities attributed to NTZ, including inhibition of chaperone-usher pilin biogenesis by E. coli and biofilm production by Staphylococcus epidermidis (15, 37, 38).
Perhaps most importantly, amixicile had lost the intrinsic cytotoxicity for immortalized cell lines noted for NTZ (14, 39). Studies presented herein indicate that amixicile was well tolerated by mice and rats receiving maximum doses (200 or 300 mg/kg, respectively) and that in vitro assays revealed no cytotoxicity to hepatocytes or measurable metabolism by liver microsome fractions obtained from human, dog or rat. Amixicile did not increase the mutation frequency in E. coli as determined by forward mutation to rifampin resistance. Bidirectional permeability studies with Caco-2 cell monolayers showed amixicile to be readily translocated in both directions, though low pH (pH 6.0 versus 7.4) appeared to affect apical uptake. Our studies showed that amixicile was not a substrate of or an inhibitor of the OAT1, OCT2, and OATP1B3 or BCRP and P-gp transporters. CYP induction studies suggested that amixicile might induce CYP1A2 and CYP3A4 at the highest concentrations tested and showed little inhibitory activity of these enzymes. Our concerns that plasma protein binding might also affect the biological activity of amixicile (noted problem with NTZ) seemed to be mitigated by MIC based studies where additions of bovine serum albumin to MIC assays had no effect (15) and by demonstrating therapeutic efficacy against H. pylori in a mouse infection model. Consistent with efficient uptake of amixicile, stool drug levels were below the level of detectability (< 0.1 μg/ml). Finally, dosing healthy mice with amixicile did not alter microbial levels of selected gut bacterial species, including Bacteroides sp, for which MIC tests had shown to be highly susceptible to this group of therapeutics (15, 19, 20). Taken together, the preclinical studies found no mitigating safety or toxicological concerns with amixicile that might preclude further development.

With the exception of metronidazole, most therapeutics used to treat CDI are minimally absorbed including vancomycin, fidaxomicin, nitazoxanide and experimental drugs like LFF571 (6, 40). This has led to the notion that therapeutics that concentrate in the gut and retain potency should be much more efficacious than systemic therapeutics. Presumably, systemic therapeutics require higher doses to achieve similar luminal concentrations. However, in an
acute CDI mouse model, systemic amoxicile showed equivalence to vancomycin and fidaxomicin
and superiority to NTZ at 5 days and superiority to all by day 14 post infection (15). Importantly,
with amoxicile and NTZ there was no relapse of CDI in any of the surviving treated animals;
whereas recurrence was observed in 70-80% of animals receiving similar doses of fidaxomicin
or vancomycin, respectively (6, 15). The apparent equivalence of a systemic therapeutic to
those of minimally absorbed therapeutics raises more fundamental questions regarding the
nature of an infection, its location and the biological action of the therapeutic at that site (i.e.,
luminal or mucosal?). Our studies would suggest that C. difficile colonizes the intestinal mucosa
and promotes local inflammation and that elimination of these organisms by treatment with
amoxicile leads to resolution of disease. This view is compatible with the notion that repopulation
of the site by resident flora, naturally or through probiotics or fecal transplants, protects against
reinfection (10, 11, 23).

To explore the nature of antibiotic action further, we tested amoxicile in a mouse model of
H. pylori infection. We had previously determined that amoxicile and NTZ were inhibitory to H.
pylori and Campylobacter jejuni in the 0.5 to 1 μg/ml range by MIC tests (14, 15). Moreover, we
had tested NTZ for efficacy against H. pylori in a C57BL/6 mouse model and like the human
studies, NTZ proved ineffective in eradicating infection (35). In the mouse model, H. pylori
reside in the gastric mucosa, but do not appreciably invade gastric epithelial cells as
demonstrated by H&E staining of stomach tissue (27). All therapeutics active against H. pylori
(e. g., metronidazole, amoxicillin, tetracycline, and clarithromycin) are systemic and must diffuse
through the gastric epithelium in order to reach the bacteria. In general, minimally absorbed
drugs are ineffective against H. pylori (36). Thus, drugs that concentrate in areas of
inflammation (serum leakage) are likely to be the most effective against H. pylori, such as MTZ,
which also tends to concentrate in gastric acid (41). Our group and others have shown that MTZ
is the most effective monotherapy in treatment of mice colonized by strains of H. pylori that are
susceptible to MTZ (42, 43). The remarkable efficacy of amoxicile as a monotherapy was
unexpected since neither amoxicillin nor clarithromycin, mainline therapeutics used for
treatment of *H. pylori* in humans, show any efficacy as monotherapies in this model (42, 43).

Based on these results, we suggest that amoxicile most likely concentrates in areas of
inflammation associated with active infections. By not accumulating in the colon, it avoids the
collateral damage to resident gut microflora that is problematic with all minimally absorbed
antimicrobials. While luminal *C. difficile* might also be spared by systemic therapeutics like
amoxicile, both washout and competition with repopulated gut microflora as suggested
previously (15) might mitigate relapse of CDI.

While amoxicile shows good efficacy against *H. pylori* in the mouse model, we have
previously reported that some MTZ resistant (MTZ') strains exhibit cross-resistance to NTZ and
consequently to amoxicile *in vitro* (32). This includes *H. pylori* strains 1061rdxAfrxA and G27rdxA
(MIC = 16 μg/ml for NTZ) and resistance is not due to mutations to *pfor*GDAB since PFOR
enzyme activity in cell-free extracts was essentially wild type and could be inhibited by amoxicile
(manuscript in preparation). These strains lack a functional RdxA NAD(P)H MTZ-reducing
nitroreductase that also exhibits potent NADPH oxidase activity (44). There is accumulating
evidence now to suggest that nitroreductases are potent scavengers of cytoplasmic oxygen and
are part of a cellular redox system that maintains an anoxic cytoplasm (44). Accordingly, loss of
RdxA function contributes to oxidative stress, leading to activation of compensatory metabolic
pathways that appear to be controlled by HsrA, a homeostatic oxidative stress regulator (45).

While we do not completely understand the underlying mechanisms that render the PFOR drug
target less essential, the phenomenon might be uniquely limited to *H. pylori*, as MTZ resistant
strains of *C. jejuni* retain susceptibility to amoxicile (14, 15). In general, MTZ resistance is rare in
the strictly anaerobic bacteria and parasites. However, a few reports of resistance in *C. difficile*
have appeared (46, 47), but since the clostridia express multiple α-keto-acid: ferredoxin
oxidoreductases, resistance would likely require additional metabolic changes. Similarly, in
Bacteroides sp, MTZ\(^r\) strains often harbor nim genes whose products nitroreduce MTZ to non-toxic ammonia (48, 49), so cross-resistance with amoxicile would be unlikely since the nitro groups of amoxicile and NTZ are not susceptible to nitroreduction (15, 32). It is important to emphasize that MTZ is both mutagenic and selective for resistance, not only to MTZ, but to other antimicrobials used therapeutically (24). While amoxicile and NTZ share the same clinical spectrum as MTZ (8, 12, 14, 15, 19, 20, 31), both their modes of action and drug targets are different.

PK studies show that amoxicile is readily absorbed and maximum plasma levels (C\(_{\text{max}}\)) of 15 and 20 \(\mu\)g/ml were obtained in rats receiving either 30 or 100 mg/kg, respectively or 179 \(\mu\)g/ml in mice receiving a single dose of 200 mg/kg. As seen in Figure 2A, rats receiving 100 mg/kg did not show the expected concentration-dependent 3-fold increase in C\(_{\text{max}}\) (assuming rapid absorption), but did show this increase when AUCs were compared. We interpret these results to suggest that the methyl-cellulose carrier caused a time release of amoxicile at the higher concentration, which also extended the time to elimination (t\(_{1/2}\)). This was not observed in the mouse study where the drug was administered in PBS in the absence of methyl cellulose. This knowledge might be useful in the future when formulations are optimized for humans. Taken together, the PK studies show that amoxicile is readily absorbed and disseminated. Based on plasma levels in both the rat and mouse studies, amoxicile readily achieved drug levels well in excess of MIC for C. difficile and H. pylori. It is also likely that plasma levels > 20 \(\mu\)g/ml might show efficacy against MTZ\(^r\) strains of H. pylori in the mouse model (MIC = 16 \(\mu\)g/ml). Studies are in progress to test this possibility.

In summary, preclinical studies indicate that amoxicile, which is in development for treatment of CDI, is well tolerated by both rats and mice, is not appreciably metabolized by liver microsome fractions, and is not cytotoxic for hepatocytes or human foreskin cells (15). PK studies indicate that the drug is efficiently absorbed and is below detectable levels in stool.
samples. While amixicile, like NTZ shows broad spectrum action against strictly anaerobic bacteria, including *Bacteroides fragilis in vitro*, amixicile did not affect gut bacterial populations of susceptible anaerobes, including species of *Bacteroides*. These studies also confirmed that bacteria lacking the PFOR drug target (Lactobacilli) are also unaffected by amixicile. In this regard, the key component microbes of probiotics (*Lactobacilli* and *Bifidobacteria*) do not contain the PFOR drug target and would not be affected by amixicile. While cross-resistance with MTZ might limit the use of amixicile for treatment of *H. pylori* in humans, further efficacy studies with resistant strains that include combination therapies and a proton pump inhibitor might overcome resistance. Finally, our studies strongly suggest that a focus on minimally absorbed therapeutics to treat CDI should be reconsidered. Clearly, a systemic therapeutic that concentrates in areas of active infection, that spares resident flora and resists mutation based drug resistance has potential application in treatment not only of CDI, but other infections where anaerobic microorganisms are involved such as periodontal disease, Crohn’s and inflammatory bowel diseases and a range of parasitic infections caused by *Cryptosporidium parvum*, *Giardia lamblia* and *Trichomonas vaginalis*.

**ACKNOWLEDGEMENTS**

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REFERENCES


Liver microsome fractions were prepared as detailed in the text. Amicicile was added at the indicated concentrations and the percent remaining compound, and any metabolic products was determined by MS. HI indicates the heat inactivated control.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Human</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μM</td>
<td>10 μM</td>
<td>1 μM</td>
</tr>
<tr>
<td>15</td>
<td>96.3 ± 4.3</td>
<td>96.5 ± 3.1</td>
<td>94.2 ± 1.8</td>
</tr>
<tr>
<td>30</td>
<td>94.5 ± 1.7</td>
<td>101 ± 5.5</td>
<td>92.4 ± 3.5</td>
</tr>
<tr>
<td>60</td>
<td>83.7 ± 8.8</td>
<td>99.9 ± 5.2</td>
<td>88.0 ± 0.4</td>
</tr>
<tr>
<td>60 HI</td>
<td>96.1 ± 3.0</td>
<td>99.2 ± 3.5</td>
<td>100.5 ± 1.2</td>
</tr>
</tbody>
</table>
Table 2. Cytotoxicity for human hepatocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μM)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>NTZ</td>
<td>10</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>NTZ</td>
<td>25</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Amixicile</td>
<td>10</td>
<td>82 ± 14</td>
</tr>
<tr>
<td>Amixicile</td>
<td>25</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>Amixicile</td>
<td>50</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>Amixicile</td>
<td>100</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>Amixicile</td>
<td>200</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>VPC16a1011</td>
<td>5</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>VPC16a1011</td>
<td>10</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>VPC16a1011</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

The percent viability of the monolayer was determined by the MTT assay at 24 h. The structures of the compounds used are presented in Figure 1.

Table 3. Induced mutation to rifampicin resistance in E. coli strain CC103.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/ml)</th>
<th>Mutation Frequency (Per 10^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amixicile</td>
<td>32</td>
<td>4.4 ± 2.7</td>
</tr>
<tr>
<td>VPC162031</td>
<td>15</td>
<td>10 ± 3.5</td>
</tr>
<tr>
<td>NTZ</td>
<td>15</td>
<td>3.8 ± 3.1</td>
</tr>
<tr>
<td>MTZ</td>
<td>15</td>
<td>21 ± 1.4</td>
</tr>
<tr>
<td>Nitrofurazone</td>
<td>2.5</td>
<td>64 ± 32</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2.9 ± 1.3</td>
</tr>
</tbody>
</table>

Forward mutation to rifampicin resistance. The average of two independent experiments were performed in triplicate.
Table 4. Plasma binding studies

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Conc. (μg/ml)</th>
<th>Percent Bound to Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>Amixicile</td>
<td>1</td>
<td>95.9 ± 0.437</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94.5 ± 0.440</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>92.1 ± 0.640</td>
</tr>
<tr>
<td>VPC16a1011</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Studies employed equilibrium dialysis. VPC16a1011 is a hydrophobic analogue of amixicile.
Table 5. Pharmacokinetic data from rat single dose experiments.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Rat No.</th>
<th>Cmax (μg/ml)</th>
<th>Tmax (hr)</th>
<th>AUClast (hr*μg/ml)</th>
<th>AUCinf (hr*μg/ml)</th>
<th>T1/2 (hr)</th>
<th>V/F (L/kg)</th>
<th>Cl/F (ml/hr/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amixicile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>20.4</td>
<td>0.50</td>
<td>107</td>
<td>107</td>
<td>1.54</td>
<td>0.621</td>
<td>279</td>
</tr>
<tr>
<td>16</td>
<td>14.8</td>
<td>0.50</td>
<td>70.6</td>
<td>70.6</td>
<td>1.65</td>
<td>1.01</td>
<td>425</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>11.2</td>
<td>0.50</td>
<td>59.6</td>
<td>59.8</td>
<td>1.39</td>
<td>1.00</td>
<td>501</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>15.5</td>
<td>0.67</td>
<td>79.2</td>
<td>79.3</td>
<td>1.53</td>
<td>0.879</td>
<td>402</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>4.6</td>
<td>0.29</td>
<td>25.0</td>
<td>24.9</td>
<td>0.13</td>
<td>0.224</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Amixicile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>18</td>
<td>18.7</td>
<td>8.0</td>
<td>298</td>
<td>331</td>
<td>6.50</td>
<td>2.84</td>
<td>303</td>
</tr>
<tr>
<td>19</td>
<td>21.8</td>
<td>1.00</td>
<td>323</td>
<td>323</td>
<td>2.04</td>
<td>0.91</td>
<td>309</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>18.6</td>
<td>0.50</td>
<td>283</td>
<td>330</td>
<td>8.25</td>
<td>3.62</td>
<td>303</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>19.7</td>
<td>3.17</td>
<td>301</td>
<td>328</td>
<td>5.60</td>
<td>2.45</td>
<td>305</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>1.8</td>
<td>4.19</td>
<td>20</td>
<td>4</td>
<td>3.22</td>
<td>1.39</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Single dose of amixicile (30 and 100 mg/kg) were administered to male rats. The results for individual rats are depicted with the means and standard deviations.
Figure Legends.

Figure 1. Chemical structures of amixicile, nitazoxanide, VPC16a1011 and VPC16b2031.

Figure 2. Time-course of plasma concentrations of amixicile following a single oral dose.
A. Male rats received 30 or 100 mg/kg drug in methyl cellulose by gavage. Each data point represents the mean plasma concentration from up to 3 rats (+ SD). B. Male mice received 200 mg/kg drug in PBS by oral gavage and at each time point, 5 mice were sacrificed for blood collection. The mean and standard deviation are presented. The data collected was used to generate the PK information presented in Table 5.

Figure 3. Therapeutic efficacy of amixicile in mice. Mice were infected with the SS1 strain of *H. pylori* and following two weeks to enable infection to manifest, mice were divided into groups with one serving as an untreated control and the others receiving one or two 20 mg/kg/day doses of amixicile or metronidazole. One week later, animals were sacrificed and stomach material was collected for bacterial enumeration. The data are reported as cfu/g of stomach material. The mean and standard deviation from 5 animals are presented. * indicates significance (p= <0.001).

Figure 4. Effect of amixicile on mouse gut microflora. Eight mice per group received either PBS or 30 mg/kk/day of amixicile by oral gavage. Primers for *Firmicutes*, *Bacteroides* sp, *Lactobacillus* sp and segmented filamentous bacteria (SFB) were quantified by 16S rRNA expression that had been normalized to total bacteria. The mean and SD are presented.
Amoxicil

Nitazoxanide

VPC16a1011

VPC16b2031