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Review

Amixicile: A Concept Therapeutic for Treatment of Chronic Anaerobic Infections

Paul S. Hoffman

Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Corresponding author: Dr. Paul S. Hoffman, Division of Infectious Diseases and International Health, University of Virginia, 409 Lane Road, Charlottesville, VA 22908, Tel: (434) 924-2893; Email: psh2n@virginia.edu

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Abstract

Anaerobic microorganisms are often associated with chronic mucosal infections including periodontal disease, inflammatory bowel diseases, and recurrent colitis caused by *Clostridioides difficile*. Management of these diseases requires a long term strategy, but available antibiotics (e.g., metronidazole) can only be used short term. Conceptually, therapeutics that control chronic inflammation would lessen the risk of associated autoimmune diseases including atherosclerosis, arthritis, type II diabetes, Crohn's and ulcerative colitis and even Alzheimer's disease. To meet this need, an antibiotic must overcome inevitable antibiotic resistance, toxicity to humans or their mitochondria and limit collateral damage (dysbiosis) to gut microbiota. This review describes attributes of amixicile (AMIX), a novel systemic therapeutic, that shows efficacy in animal models for treatment of C. *difficile* colitis and gastric infections caused by *Helicobacter pylori*, while limiting collateral damage to gut microflora. Together with the apparent absence of drug resistance, toxicities, and drug metabolism, might qualify amixicile for consideration as a long term therapeutic for management of chronic and acute anaerobic infections.

Key words: Anaerobic infections, Amixicile, antibiotic resistance, chronic infection and Clostridioides difficile

Introduction

Amixicile (AMIX) is a second generation derivative of nitazoxanide (NTZ) that is in early stages of clinical development for treatment of infections caused by *Clostridioides (Clostridium) difficile* (CDI) [1-3]. NTZ is a US Food and Drug Administration (FDA)-approved treatment for intestinal infections caused by *Giardia* and *Cryptosporidium* and shows some efficacy in humans for treatment of C. *difficile* infections [4-6]. However, NTZ has several pharmacologic shortcomings (poor pharmacokinetics and drug metabolism) and worrisome "off target" activities that limit its potential [5-11]. Amixicile (AMIX), like NTZ, inhibits the essential enzyme pyruvate: ferredoxin oxidoreductase (PFOR) and related members of the α-keto-acid oxidoreductase family found in obligate anaerobic bacteria, anaerobic human intestinal parasites, and in members of the epsilonproteobacteria (*Campylobacter* and *Helicobacter*) [1,3,12]. This target is not found in humans, mitochondria or in aerobic and facultative anaerobes which utilize pyruvate dehydrogenase (PDH) in the catabolism of pyruvate [1,12]. In contrast to NTZ, AMIX is a systemic (efficiently absorbed) compartmentalized therapeutic that is slowly eliminated via the renal system and is not excreted in feces and exhibits no effect on the gut microflora of healthy mice [2]. Importantly, by replacing the acetyloxy group on the benzene ring of NTZ with propylamine (Figure 1), PFOR target specificity is improved while drug metabolism and noted "off target" activities are eliminated [1-3]. This review provides an update on AMIX, its novel mode of action, antimicrobial spectrum, and potential clinical use in management of chronic anaerobic infections, including prophylaxis and prevention of CDI.

Amixicile may Escape Mutation-Based Drug Resistance

One of the greatest challenges to development of any new class of antimicrobials is finding drug targets and inhibitory mechanisms capable of escaping the inevitable development of antibiotic resistance. The vast majority of synthetic antimicrobials are often defeated by mutation-based drug resistance [13]. One of the fundamental questions we addressed early on with the PFOR drug target is why "Mother Nature" had not found this target? While anaerobe-selective therapeutic metronidazole (MTZ) (Figure 1) is reductively activated by the reduced ferredoxin component of PFOR [14], no other inhibitors, natural or synthetic, have been found to interfere with the catalytic mechanism of this enzyme. Interrogation of the NTZ structure (Figure 1) revealed the 5-nitrothiazole moiety was responsible

for biological activity and that a wide variety of heterocyclic tail compounds could replace the benzene ring of NTZ [3,15]. These studies determined that the 5-nitrothiazole of NTZ and AMIX is anionic under physiological conditions and it is the nitro-anion (Figure 2) that abstracts a proton (hydrogen ion) from the activated vitamin B1 cofactor (thiamine pyrophosphate, TPP) of PFOR [3,12,15]. The kinetics of proton abstraction can be followed spectrophotometrically and kinetic studies revealed that NTZ/AMIX were over two orders of magnitude more efficient than pyruvate in binding activated TPP [12]. Proton abstraction inactivates the catalytic cycle of PFOR, prevents conversion of pyruvate to CO2, Acetyl CoA and reducing equivalents, and is dependent on a functional enzyme and vitamin cofactor [12]. Several caveats that make PFOR an attractive drug target are: 1) it is a highly conserved ancient enzyme; 2) the amino acids associated with TPP function are conserved among the entire family of PFOR enzymes (Figure 2), regardless of the number of subunits composing the holoenzyme; 3) these enzymes are essential for viability; and 4) TPP is uniquely bound and contorted within the catalytic pocket, a feature not found with TPP in other enzymes such as PDH [1,3,12,15]. The "perfect storm" for this target and inhibitory mechanism is that mutations to the enzyme are lethal and mutations that alter TPP, itself a small molecule, are also considered to be functionally lethal (12). Laboratory selections for NTZ or AMIX resistant mutants from C. *difficile, Treponema denticola, Porphyromonas gingivalis* or even *Helicobacter pylori* have not delivered any mutants [1,2,3,14,16,17]. Importantly, there appear to be no reports of drug resistance from clinical use of NTZ or among laboratory collections of anaerobic bacteria that includes species of Bacteroides and Clostridium [8]. Based on in vitro MIC testing, AMIX and NTZ are equally potent against both Gram negative and positive bacteria suggesting these small molecules efficiently enter bacteria. For most antibiotics, their lethal effect is thought to provide a powerful selective pressure, enabling outgrowth of drug resistant variants. In contrast, the phenotype for the inhibitory effect of AMIX on susceptible bacteria is one of starvation, resembling that caused by growth in a nutrient deficient environment. Bacteria stop growing and eventually die.

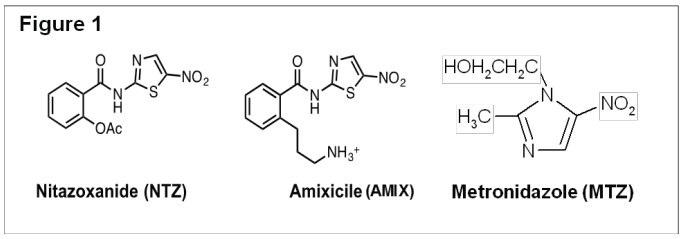

Figure 1. Chemical structures. Amixicile was derived from NTZ by replacing the OAc group with propylamine. Primary amines are thought to improve absorption by Gram negative bacteria. Metronidazole is a redox active prodrug that is activated by nitroreduction via the ferredoxin component of PFOR.

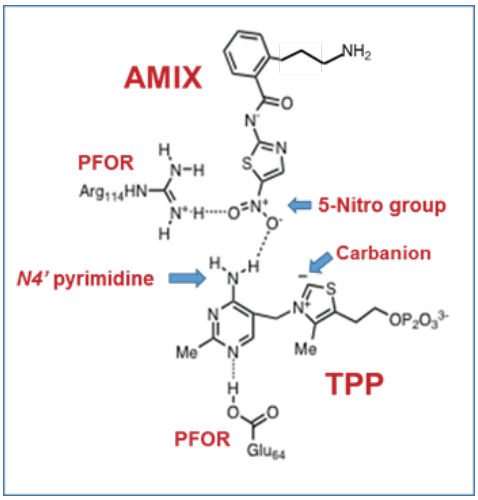

Figure 2. Mode of Action for AMIX. Within the PFOR active center, the indicated carbon (arrow) in the thiazole ring of TPP is converted to a carbanion (where pyruvate binds) by transfer of the proton to the N4' pyrimidine of TPP. The anionic 5-nitro anion of AMIX and NTZ is attracted to the N4' proton of the aminopyrimidine [3]. Proton abstraction results in inactivation of the PFOR catalytic cycle. Protonation of AMIX and NTZ eliminates resonance within the 5-nitrothiazole ring which can be visualized by a color change from yellow to colorless. AMIX and NTZ are reactivated by rapidly losing the proton to water at physiological pH [12]. Arginine-114 and Glutamate-64 are conserved amino acids within the catalytic center and required for PFOR and vitamin function.

Cross Resistance

We have found no cross-resistance with MTZ, as MTZ-resistant strains of C. *difficile* and *Bacteroides fragilis* retain susceptibility to AMIX, which might be important in the future as MTZ-resistance in the anaerobes is a growing problem. Our studies have not fully ruled out the possibility that second-site mutations that, for example, alter PFOR expression levels or activate compensatory metabolic pathways might increase drug resistance (a form of drug tolerance), much like upregulation of efflux pumps contributes to quinolone resistance [2]. Preliminary studies with MTZ-resistant strains of *H. pylori* suggest a compensatory metabolism strategy (tolerance) that is dependent on loss of function mutations to the RdxA MTZ-reducing nitroreductase and not in the PFOR enzyme [2,19,20]. It is noteworthy that because MTZ is highly mutagenic and selective, forward mutation to MTZ-resistance can readily be selected from susceptible strains exposed to MTZ, while the reverse (exposure to NTZ or AMIX) does not, consistent with the absence of a selectable phenotype [14,21].

Pharmacokinetics, ADMET and Tolerability

While antimicrobials are generally active against microbes in the micromolar range, they are administered at relatively high concentrations (100 mg to several grams per day). With systemic antimicrobials, absorption and serum levels above the minimal inhibitory concentration (MIC) are crucial for therapeutic efficacy. AMIX is efficiently absorbed and in two studies, one in rats and one in mice, AMIX was found to achieve good AUC/MIC, a C_{max} essentially of infinity (oral administration) and slow renal elimination and in drug tolerance studies AMIX was well tolerated at 300 mg/kg (24 grams per dose in human equivalents) in rats and mice [2]. Unlike NTZ, AMIX was not metabolized by human, dog or rat liver microsomal fractions and showed no toxicity to various mammalian cell lines [2]. While AMIX was weakly positive by Ames test (similar to NTZ), the genotoxicity studies were negative [2]. In serum binding assays, AMIX exhibited an order of magnitude less serum binding than NTZ and in contrast to NTZ, AMIX potency was unaffected by increasing serum concentrations in MIC tests [1,2,16]. Given the absence of "red flags" in preclinical development, the next stage of pre-IND

development requires completion of maximum tolerable dose studies in dogs and rodents and the requisite toxicology, cardiovascular and other physiological tests to assess safety. Since one of the potential indications for AMIX is treatment of chronic infections, it will be important to evaluate the long term stability, tolerability and safety in animal models, including non-human primates prior to first in human studies.

Efficacy Studies and Spectrum

AMIX bucks the conventional wisdom that therapeutics targeting C. *difficile* need to be non-absorbable (concentrate in the gut) [22]. While MTZ is an exception (systemic therapeutic), all the other antibiotics used to treat CDI (vancomycin, fidaxomicin, rifaximin, and NTZ) and ones in clinical development (Ridinilazole) concentrate in the gut (poorly absorbed). It is also noteworthy that the majority of ongoing CDI clinical trials do not involve new antimicrobials, but rather biologics and fecal material transplant (FMT) therapeutics (ClinicalTrials.gov). Apparently, none of the current antibiotics is as good clinically as FMT in preventing recurrence of CDI [23]. This is because antibiotics that concentrate in the gut are not sufficiently selective to avoid collateral damage to gut flora, which often includes beneficial probiotic microbes *Bifidobacterium* sp and *Lactobacillus* sp associated with colon health and protection from CDI [24]. In head to head comparative studies with vancomycin and fidaxomicin in an acute mouse CDI infection model, AMIX was slightly less effective (not statistically significant) than the standard therapies at 5-days post infection, likely a result from quicker action of non-absorbed drugs in this acute CDI model [1]. However, AMIX proved superior to vancomycin and fidaxomicin at 14-days post infection (100% survival of day 5-mice) compared with 30% and 20% survivors with vancomycin and fidaxomicin treated animals, respectively at 14 days [1]. Both vancomycin and fidaxomicin exhibit collateral damage to Gram positive gut flora including the Bifidobacteria and Lactobacilli. AMIX prevented relapse in this model because it does not concentrate in the gut and thereby enables the protective gut microflora to rebound during the course of antibiotic therapy [1]. It is important to note that neither NTZ nor AMIX inhibit growth of the Bifidobacteria and Lactobacilli since these bacteria lack the PFOR drug target [1]. The distinguishing difference between NTZ and AMIX is that NTZ mostly concentrates in the gut where it contributes to dysbiosis and to clinical relapse [6]. Healthy mice fed 200 mg/kg of AMIX showed no detectable AMIX in fecal pellets and mice fed 30 mg/kg for 3 days showed no change in composition of the gut microbiome, confirming the lack of collateral damage [2]. Our studies found AMIX to be equally potent in vitro against the hypervirulent binary toxin-producing strains (NAP1/BI/027) of *C. difficile* that are commonly acquired in hospital settings [1]. Thus, AMIX shows promise for use in primary treatment, as well as in prevention of CDI in high risk setting such as hospitals and nursing homes.

AMIX Concentrates in Mucosal Pockets of Inflammation

Perhaps the obvious question to ask is how can a systemic therapeutic that does not concentrate in the gut, show efficacy against CDI? Like most toxigenic diarrhea-producing pathogens, *C. difficile* infects gut epithelial cells where TcdA and TcdB toxins (or binary toxin) is released locally to produce tissue damage (pseudomembranous colitis) that promotes inflammation [25]. We speculated that AMIX, by virtue of its binding serum proteins might concentrate locally in these pockets of inflammation via serum leakage [1,2]. To test this hypothesis, we evaluated the efficacy of AMIX against another mucosal pathogen *H. pylori*, in a mouse infection model. While never published, we previously examined the efficacy of NTZ against *H. pylori* in this model nearly 20 years ago and found no efficacy when compared with the gold standard MTZ. In contrast, AMIX showed equivalence with MTZ in this model [2]. Since MTZ concentrates in gastric tissue, we reasoned that AMIX must also, supportive of the localized mucosal inflammation hypothesis.

A third animal study provides additional evidence for AMIX accumulation in sites of inflammation. In this study AMIX was compared with NTZ for efficacy against *Cryptosporidium parvum* in a protein malnourished mouse model [26]. In the malnourished mouse, decreasing weight loss is common and when infected with *Cryptosporidium,* animals show accelerated weight loss [26]. While NTZ is the only FDA-approved drug for treatment of *Cryptosporidium* infections in humans, the drug does poorly in immunocompromised children and HIV patients [27]. In general, NTZ shows poor efficacy in various animal models [28]. In the malnourished mouse model, NTZ showed no efficacy, whereas similar concentrations of AMIX showed efficacy [26]. On further examination, AMIX, but not NTZ, reversed weight loss in malnourished uninfected control mice. Even more confounding was that AMIX showed no *in vitro* inhibitory activity against *Cryptosporidium* [26]. Remarkably, *Cryptosporidium* and related species contain a hybrid PFOR, one that is fused with Cytochrome P450 [29]. Cytochrome P450 is not a cytochrome, but contains flavodoxin and NADPH oxidase and uniquely couples pyruvate oxidation with reduction of NADP [26,29]. Based on PFOR docking simulations [3] it is likely that neither drug inhibits this variant of PFOR and that the action of NTZ is most likely against a different target. So what is going on? The hallmark of environmental enteropathy in malnourished children and in protein-starved animal models is an overpopulation of anaerobes and enteric bacteria in the small intestine and duodenum where they promote inflammation which leads to loss of barrier function and malabsorption [30-33]. Based on the serum leakage hypothesis, we proposed that AMIX, by tamping down the anaerobic component in the small intestine, reduced inflammation, leading to restoration of barrier function and, by reversing weight loss, enabled healthy mice to self-cure [26]. One of the limitations of this study is that high doses of NTZ and AMIX (100 mg/kg) were used, and studies are in progress to determine whether lower drug concentrations (10 – 20 mg/kg) might prove as effective. Since environmental enteropathy in malnourished children during the first two years of life is associated with growth stunting and cognitive impairment, which are irreversible, perhaps early therapeutic intervention with AMIX might prove beneficial [33].

Chronic Infections and Autoimmune Diseases

Chronic inflammation is a recognized risk factor in the development of many autoimmune diseases including rheumatoid arthritis, atherosclerosis, type II diabetes, Crohn's disease, Parkinson's and even Alzheimer's disease [34-37]. Life-long infections of the gastric mucosa (chronic gastritis) caused by *Helicobacter pylori,* chronic oral anaerobic infections (gingivitis and periodontitis) and inflammatory bowel diseases (ulcerative colitis and Crohn's) are all challenging to manage clinically. While in theory these diseases can be managed by antibiotic interventions, long term treatment with antibiotics is not recom-

mended because of the risk of drug resistance, adverse effects and disruption of gut microflora (dysbiosis). Gut dysbiosis adversely affects human immune responses, effectiveness of cancer therapeutics, efficacy of vaccines, and can lead to serious intestinal infections such as *C. difficile* colitis (CDI) and colon cancer [38,39]. Repeated rounds of antibiotic therapy is often unsuccessful in management of these chronic diseases. Perhaps, maintenance therapy with AMIX over many months might ameliorate these infections and enable damaged tissue and resident flora to reestablish [16,17]. For other hospital acquired diseases caused by *C. difficile* (CDI), prophylaxis and post discharge maintenance therapy for several months might prevent and eventually eliminate this pathogen from hospital and nursing home environments. Similarly, AMIX is under consideration for management of periodontal disease (PD), as it shows selective potency against the "red complex" anaerobes associated with this disease [16,17]. With Crohn's and other gut mucosal inflammatory disorders, prevention of relapses might be possible and similarly in malnourished children (environmental enteropathy), restoration of barrier function by tamping down the associated anaerobes might improve health by preventing stunting and cognitive impairment [26]. Human studies are required to evaluate whether therapeutics like AMIX could show similar benefit in IBD as TNF-α monoclonal antibodies (imunotherapeutics), without immunosuppression contraindications [40].

Concluding Remarks

The growing problem of antimicrobial resistance and emergence of superbugs (multi-drug resistant Gram negative pathogens) challenge our health care industry. Meeting this challenge is complex because in general, most bacterial infections, including many caused by superbugs, are treatable with generic antibiotics and the new medicines are limited by prohibitive costs compared to those of generics [41]. While the WHO, CARB-X and other agencies support development of therapeutics against a short list of priority pathogens because of their high mortality rates, from a business perspective, this is a small market (less than $100 million in sales per year) and consequently considered unattractive to investment [42]. This includes the CDI drug fidaxomicin (~$3,000 per treatment) which earned ~$50 million in sales in 2018 [42]. The headwinds and lack of enthusiasm for new antimicrobials outside the priority list window is even greater, despite the potential to reach larger markets. AMIX would compete with MTZ which enjoys a large market share because it is very cheap (~$22.00 dollars a treatment), but has serious side effects (Black Box warning from FDA) and long term use is not recommended. A therapeutic such as AMIX, if clinical trials validate its safety, could gain market share as a preventative, not only for CDI, but for PD, IBD and environmental enteropathy, that when combined would be a multi-billion-dollar market. One might envision that such medicines might be prescribed for many months or even life for management of chronic infections in certain high risk groups, much like statins, blood pressure and type II diabetes medicines are currently prescribed. Conceptually, thinking outside the box of traditional antimicrobial development, might lead to new perspectives regarding other major human diseases, including many cancers, where an underlying microbial etiology is suspected. Our growing understanding of the significant role of our microbiome to our health and how dysbiosis contributes to many diseases represents a starting point in development of therapeutic interventions that just might improve our collective health.

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