Repurposing of Meropenem and Nadifloxacin for Treatment of Burn Patients?

Joseph G. Moloughney[1*], Janice D. Thomas[1*], Christie E. Costa[2], Karla S. Bullon[2], James Spencer[3], Jeffrey H. Toney[4†]

Address

[1]Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, USA, [2]Department of Biological Sciences, Kean University, Union, NJ 07083, USA,[3]Department of Cellular and Molecular Medicine, University of Bristol, Bristol BS8 1TD, [4]College of Natural, Applied and Health Sciences, Kean University, Union, NJ 07083, USA

[*]These authors contributed equally to this work.

[†]To whom correspondence should be addressed: E-mail: jetoney@kean.edu

The escalating number of multidrug resistant pathogens has demanded the swift development of new and potent antibiotics (ref. 2). Metallo-β-lactamases (MBLs) continue to evolve, rendering the latest generation of carbapenem antibiotics useless (ref. 8). SPM-1, a recently discovered MBL, was isolated from a juvenile leukemia patient residing in a hospital in San Palo, Brazil just prior to the patient succumbing to septicemia brought on by *Pseudomonas aeruginosa* expressing SPM-1 (ref. 8). Screening of the Johns Hopkins Compound library of 1,514 FDA or FAD approved drugs (ref. 1) identified a novel SPM-1 inhibitor that is synergistically compatible with meropenem. Using clinically achievable concentrations, meropenem coupled with nadifloxacin inhibits *Pseudomonas aeruginosa* expressing SPM-1. This

shotgun approach to new drug discovery provided a prompt solution to the grave problem of antibiotic resistant pathogens that are thriving in hospitals today.

The challenges associated with drug development are vast and have been well documented, but a straightforward, cost-effective alternative already exists: screening existing drugs for novel uses. For example, a screen of the Johns Hopkins Clinical Compound Library (JHCCL; a publicly available collection of >1,500 existing drugs) found astemizole, a common over the counter antihistamine, to effectively kill the *Plasmodium* parasite responsible for causing malaria, including chloroquine resistant strains (ref. 1). Here we apply this strategy to target bacterial antibiotic resistance.

New antibiotics are needed to combat the increasing number of resistant bacterial strains that are prevalent in hospitals today. Unfortunately, the discovery and development of new antibiotics is not meeting the clinical need due to increasing resistance (for a review see ref. 2) and the growing numbers of immunocompromised individuals with HIV, patients undergoing cancer chemotherapy and burn wound patients are now at risk from hospital-acquired (nosocomial) infections. It is estimated that 50% of all deaths caused by burns are the result of intractable bacterial infections with a high occurrence in patients infected with *P. aeruginosa* (ref. 3). *P. aeruginosa* is a versatile environmental organism that can subsist over a wide range of nutrient sources and conditions and that can form biofilms in which a significant portion of the bacterial population can produce exopolysaccharides that prohibit phagocytosis (ref. 4). These characteristics allow *P. aeruginosa* to flourish in hospital environments, making it the second most common nosocomial bacterial pathogen. Nosocomial *P. aeruginosa* strains

are often resistant to multiple antibiotics (ref. 5) resulting in chronic infections with the highest mortality rate (ref. 6).

One mechanism of antibiotic resistance is the hydrolytic inactivation of β-lactam antibiotics by MBLs (ref. 7). MBL enzymes such as SPM-1, originally identified from a *P. aeruginosa* clinical isolate (ref. 8), are class B β-lactamases that confer resistance in bacteria to a wide range of β-lactam antibiotics, including carbapenems. β-lactams remain the most widely prescribed class of antibiotics while the carbapenems are often the most (or only) effective antibiotics for treating resistant organisms such as *P. aeruginosa*. New therapeutic strategies for the treatment of infections by antibiotic-resistant bacteria, particularly carbapenem-resistant *P. aeruginosa*, in burn centers and hospital settings in general are urgently required (ref. 9).

One approach to extend the utility of carbapenems and other β-lactam antibiotics is through combination therapy. Examples include augmentin™ (amoxicillin-potassium clavulanate) and Unasyn™ (ampicillin-sulbactum) that combine a β-lactam antibiotic with a potent β-lactamase inhibitor. However, such treatments are ineffective against many β-lactamases, including MBLs such as SPM-1. To date, only three modest SPM-1 inhibitors have been reported and are not clinically useful (ref. 10). The JHCCL collection contains 1,514 existing drugs that are either FDA approved and/or Foreign Approved Drugs (FAD) (ref. 1). Using the cephalosporin nitrocefin as a substrate, the JHCCL was screened for inhibitors of SPM-1. Out of three compounds that were defined as potential leads, nadifloxicin, a topical fluoroquinolone used in creams to treat *acne vulgaris* and bacterial skin infections, was studied further. Nadifloxacin has broad-spectrum antibacterial activity against aerobic Gram-positive, Gram-negative and

anaerobic bacteria, and exhibits a K_i *of* 9.8 $\pm$ 2.3 μM (~ 3.5 μg/mL) for SPM-1 displaying a mixed mode of inhibition (ref. 11).

The ideal MBL inhibitor should reverse carbapenem resistance in a clinically relevant bacterial strain. The carbapenem meropenem is indicated as empirical therapy in both adults and children with a broad range of serious infections including complicated skin and skin structure infection (cSSSI) (ref. 12). In one of the largest studies conducted to date of hospitalized patients with cSSSI, 500 mg of meropenem administered every 8 hrs. was found to be safe and effective (ref. 13). However, such therapy would be ineffective in patients harboring carbapenem resistant bacterial strains such as *P. aeruginosa*. *P. aeruginosa* expressing SPM-1 exhibits a minimum inhibition concentration (MIC) for meropenem of 500 μg/mL, approximately a 100-fold increase in resistance compared to wild type *P. aeruginosa* strain PA01. The SPM-1 inhibitor nadifloxacin exhibits profound synergy with meropenem at clinically achievable concentrations near the K_i value (**Fig. 1**) in a *P. aeruginosa* strain expressing SPM-1.

Fluoroquinolones are effective antibacterial agents which are generally well tolerated and in some cases, have been life saving (ref 14). Nadifloxacin targets DNA gyrase, while carbapenems target biosynthesis of the bacterial cell wall. Thus, the probability of emerging antibiotic resistance against both mechanisms is very low. Synergy of cephalosporins and fluoroquinolones in *P. aeruginosa* has been reported (ref. 15) but not in highly resistant strains to carbapenems as described in this report. Of the 1,514 compounds in the JHCCL, 19 belong to the quinolone family; only nadifloxacin exhibited significant SPM-1 inhibition indicating a highly specific interaction with the enzyme target. The capability of nadifloxacin, an antibiotic already approved for clinical

use, to reverse meropenem resistance in *P. aeruginosa* revealed in this study suggests a new combination therapy for potentially lethal infections in burn patients.

Methods

Screening of JHCC

The Johns Hopkins Clinical Compound Library (JHCCL) version 1.0 containing 1,514 drugs was screened for inhibitory activity against purified SPM-1. Lead compounds, defined as ≥ 50% inhibition of SPM-1 at a final concentration of 40 μM, were identified by measuring the hydrolysis of the chromogenic substrate nitrocefin (Calbiochem, San Diego, CA, USA), at 490 nm, using a PowerWave^{TM} microplate spectrophotometer (BioTek, Winooski, VT, USA). All assays were carried out in 50 mM cacodylic acid buffer (100 μM $ZnCl_2$, 0.1% BSA, pH 7.0). SPM-1 enzyme (final concentration of 10 nM) was pre-incubated with each compound in the JHCCL (final concentration 40 μM) for 30 minutes at 25°C. Initial velocities were measured following the addition of substrate (final concentration 20 μM). The final concentration of DMSO in the assay did not exceed 2.4% (vol/vol).

K_i Determination

The K_i value for nadifloxacin was calculated by varying substrate and inhibitor concentrations using Lineweaver-Burk plots of 1/V versus 1/S. Data was fitted using SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA). The reported K_i value represents three independent experiments with four replicates in each experiment.

Synergy experiments

P. aeruginosa, ATCC strain PA01, was transformed with the SPM-1(pK18) clone (ref. 16 and 17) by electroporation in 0.2 cm cuvettes at 2.5 kV, 25 μF and 200 Ω to generate

a pulse of approximately 6 msec. After electroporation, the cells were resuspended in 1 mL of LB broth and incubated at 37ºC for 1 hour with vigorous shaking. 500 µl of cell suspension was plated on LB agar supplemented with meropenem (50 µg/mL) for transformant selection, and incubated at 37ºC for 24 hrs (ref. 18). The meropenem resistant colonies were collected separately and inoculated into LB broth supplemented with meropenem (50 µg/mL).

Minimum inhibitory concentrations (MIC) were determined using a checkboard microtitration in a 96-well plate format. Briefly, nadifloxacin (LKT Laboratories, St. Paul, MN, USA) and meropenem (Sequoia Research Products, Oxford, UK) at varying concentrations were added to a 1:20 dilution of overnight culture. The plates were incubated for 18 hrs. at 37ºC with shaking. MIC was determined by measuring OD at 650 nm. To calculate synergy, nadifloxacin and meropenem were tested alone. The fractional inhibitory concentration (FIC) was calculated using;

Combined FIC of drug A + B ≤ 0.5 for synergy (inhibitory concentration of A divided by MIC of A) + (inhibitory concentration of B divided by MIC of B).

Figures

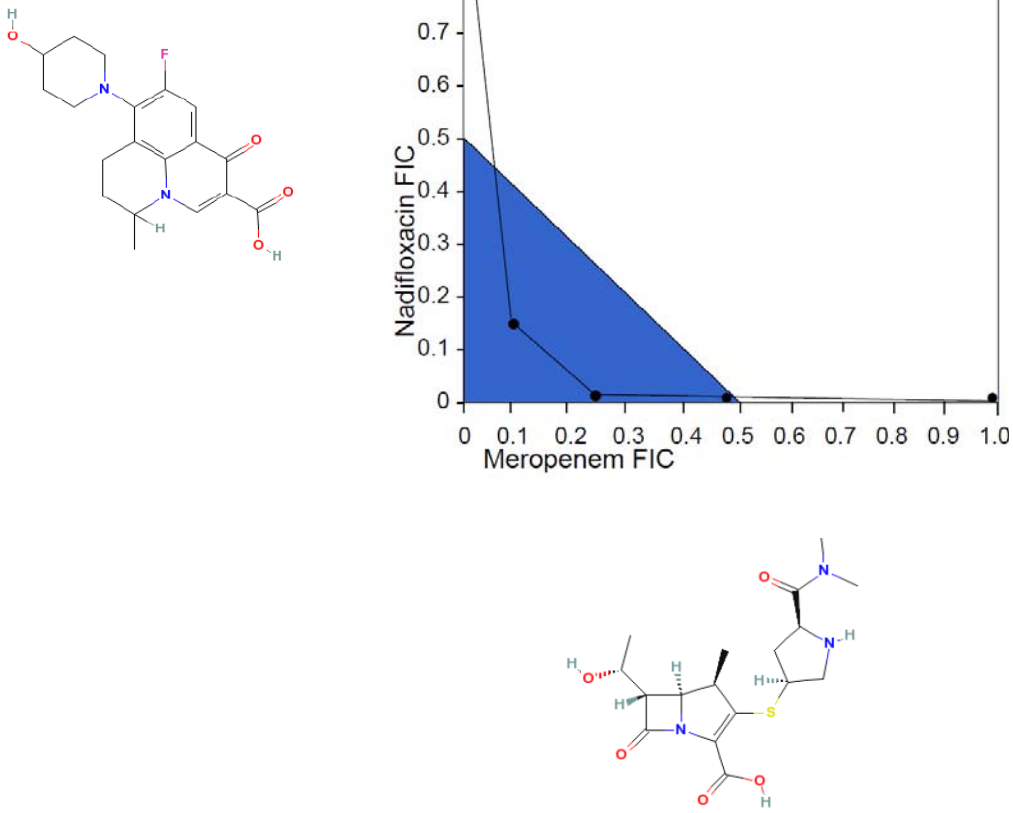

Figure 1. Isobologram of meropenem in combination with nadifloxacin against *P. aeruginosa* expressing SPM-1 demonstrating synergy. Chemical structures of meropenem (x axis) and nadifloxacin (y axis) are shown. FIC represents fractional inhibitory concentration.

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Author Contributions

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