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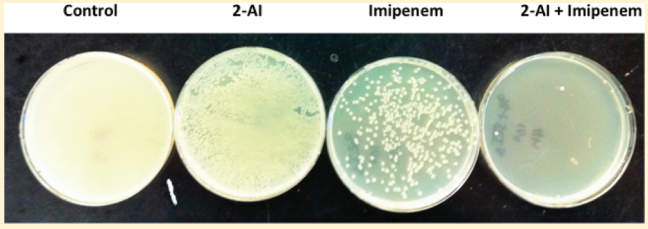
Small Molecule Suppression of Carbapenem Resistance in NDM-1 Producing *Klebsiella pneumoniae*

Roberta J. Worthington, Cynthia A. Bunders, Catherine S. Reed, and Christian Melander*

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States

Supporting Information

ABSTRACT: The already considerable global public health threat of multidrug-resistant Gram-negative bacteria has become even more of a concern following the emergence of New Delhi metallo-β-lactamase (NDM-1) producing strains of *Klebsiella pneumoniae* and other Gram-negative bacteria. As an alternative approach to the traditional development of new bactericidal entities, we have identified a 2-aminoimidazole-derived small molecule that acts as an antibiotic adjuvant and is able to suppress resistance of a NDM-1 producing strain of *K. pneumoniae* to imipenem and meropenem, in addition to suppressing resistance of other β-lactam nonsusceptible *K. pneumoniae* strains. The small molecule is able to lower carbapenem minimum inhibitory concentrations by up to 16-fold, while exhibiting little bactericidal activity itself.



KEYWORDS: *NDM-1, Klebsiella pneumoniae, antibiotic adjuvant, resistance suppression, 2-aminoimidazole*

The emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant global public health threat. Drug-resistant bacterial infections cause considerable patient mortality and morbidity, and rising antibiotic resistance is seriously threatening the vast medical advancements made possible by antibiotics over the past 70 years.[1] This situation is so dire that the Infectious Diseases Society (ISDA) has issued a call to action from the biomedical community to deal with the multidrug-resistant (MDR) bacterial threat.[2]

The majority of recent efforts have focused on strategies to deal with MDR Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). In fact, the only two new antibiotic classes introduced into the clinic in the last two decades (daptomycin and linezolid) are Gram-positive selective. There have not been the same research efforts and successes directed toward the development of strategies to deal with the increasing prevalence of MDR Gram-negative bacterial infections.[3] *Klebsiella pneumoniae* is among the most frequently observed nosocomial Gram-negative bacteria worldwide and causes numerous infections including urinary tract infections, pneumonia, and intra-abdominal infections.[4] *K. pneumoniae* has caused increasing concern in recent years, as strains have become resistant to virtually all antibiotics, through both mutations in chromosomally encoded genes and acquisition of genes from mobile plasmids and integrons.[5] Extended-spectrum β-lactamase (ESBL) producing *K. pneumoniae* strains have become endemic in hospitals across many geographical regions. ESBLs confer resistance to third generation cephalosporins such as ceftriaxone, cefotaxime, and ceftazidime, in addition to monobactams such as aztreonam.[6] Infections caused by ESBL producing *K. pneumoniae* have been successfully treated in the past with carbapenem antibiotics, typically one of the treatments of last resort for MDR *K. pneumoniae*.[7] However, isolation of carbapenemase producing strains, along with strains possessing porin deletions, has been increasingly reported, thus rendering these newer MDR *K. pneumoniae* strains recalcitrant to carbapenem therapy.[5]

In 2008, a new plasmid-encoded metallo-β-lactamase was identified from a *K. pneumoniae* clinical isolate recovered from a patient hospitalized in New Delhi and named New Delhi metallo-β-lactamase (NDM-1).[8] NDM-1 is not inhibited by current β-lactamase inhibitors and is able to inactivate all β-lactams except aztreonam; however, NDM-1 encoding plasmids coharbor a number of widely varying resistance determinants, rendering bacteria carrying this plasmid resistant to almost all current antibiotics. Of significant concern is that NDM-1 producers are already becoming highly prevalent, and NDM-1 has been observed in clinical isolates of the opportunistic bacterium *Acinetobacter baumannii* and in strains of *Escherichia coli* and other Enterobacteriaceae. Current therapy for carbapenem-resistant *K. pneumoniae* clinical isolates is limited to tigecycline or polymyxins such as colistin; however, development of resistance to both of these antibiotics has been encountered during treatment regimens.[9]

While the development of new antibiotics is one approach for the treatment of MDR *K. pneumoniae*, the fact remains that only two new classes of antibiotics have been introduced into the clinic over the last two decades.[10] Furthermore, bacteria invariably develop resistance to any introduced therapy that

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relies solely upon a bacteriostatic/bactericidal mechanism. For example, daptomycin was introduced into the clinic in 2003, and daptomycin-resistant strains were identified less than a year later.[11] Alternative approaches to controlling bacterial infections are therefore sorely needed.[12]

We recently reported that 2-aminoimidazole (2-AI) **1** (Figure 1) has the ability to lower the minimum inhibitory

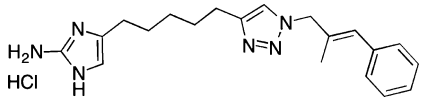
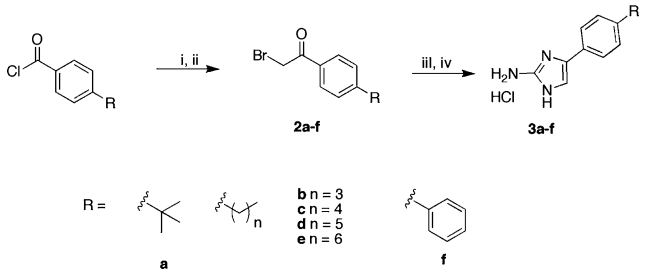


Figure 1. Small molecule **1** is able to suppress MDRAB and MRSA antibiotic resistance.

concentration (MIC) of certain antibiotics against MDR *A. baumannii* (MDRAB) and MRSA.[13] Given the unmet medical need associated with NDM-1 *K. pneumoniae*, we initially screened compound **1** for antibiotic resistance suppression activity against the NDM-1 producing strain of *K. pneumoniae* available from the ATCC (BAA-2146). Unfortunately, this compound was found to possess only marginal activity.

Subsequently, we screened ∼50 compounds with known biological activities from our in-house 2-AI library for the ability to suppress resistance of *K. pneumoniae* to imipenem and meropenem using a modified broth microdilution protocol.[14] In this assay, the MIC of each 2-AI derivative is initially determined in cation-adjusted Mueller Hinton broth (CAMHB). The MICs of imipenem and meropenem are then subsequently determined in CAMHB supplemented with the 2-AI at a concentration of ≤30% of the MIC. It is our experience that our 2-AI derivatives, at a concentration of 30% of the MIC, exhibit very little microbicidal activity, thus allowing us to attribute suppression of antibiotic resistance to nonmicrobicidal effects of the compounds. From this screen, we identified a series of analogues of 2-AI-aryl compounds[15] that are members of our library (Scheme 1) and were shown to

Scheme 1. Synthesis of 2-AI Aryl Series 3a−f[a]



[a]Reagents and conditions: (i) CH_2N_2, Et_2O, 0 °C, 1 h. (ii) Concentrated HBr, 15 min. (iii) $BocHNC(NH)NH_2$, DMF, room temperature, 72 h. (iv) TFA, CH_2Cl_2, room temperature, 2 h.

lower the MICs of imipenem and meropenem against the NDM-1 producing strain of *K. pneumoniae*. This synthesis of this series is depicted in Scheme 1 and commenced with conversion of the commercially available *para*-substituted benzoyl chlorides to the corresponding α-bromoketones, by treatment with diazomethane followed by HBr. The α-bromoketones were then cyclized with Boc-protected guanidine to form the 2-AI heterocycle. Subsequent TFA-mediated deprotection and counterion exchange afforded 2-AI HCl salts **3a−f**, which were used for biological testing.

Analogues with straight chain alkyl substituents of intermediate length (five or six carbons) on the phenyl ring were found to be considerably more active than those possessing a butyl or heptyl chain. The more bulky *t*-butyl and phenyl substituents exhibited very little activity (Table 1).

Table 1. NDM-1 *K. pneumoniae* Carbapenem MICs in the Presence of Compounds 3a−f

	MIC (μg/mL)	
compd (MIC/concn tested)	imipenem	meropenem
no compd	64	256
3a (200/50 μM)	64	128
3b (200/50 μM)	32	128
3c (100/30 μM)	8	32
3d (100/30 μM)	4	16
3e (200/50 μM)	32	64
3f (>200/50 μM)	64	128

The lead compound of this series, compound **3d**, was able to reduce the MICs of both imipenem and meropenem against the NDM-1 producing *K. pneumoniae* strain by 16-fold (from 64 to 4 μg/mL for imipenem and from 256 to 16 μg/mL for meropenem) at a concentration (30 μM) that was shown to exhibit very little microbicidal activity by colony count analysis (Figure 2). Increasing the concentration of compound **3d** to 40

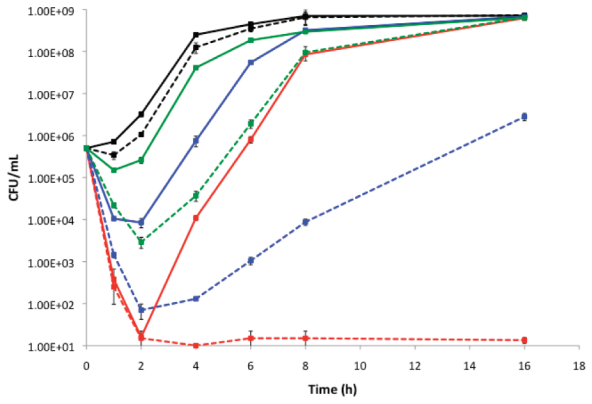


Figure 2. Time−kill curves for combinations of imipenem (IMP) and compound **3d** against *K. pneumoniae* BAA-2146. Solid lines indicate the absence of compound **3d**, and broken lines indicate the presence of compound **3d** (30 μM). Black = control, red = 32 μg/mL IMP, blue = 8 μg/mL IMP, and green = 2 μg/mL IMP.

and 50 μM resulted in MIC reductions of 64- and 256-fold, respectively, for imipenem, taking the MIC below the new CLSI breakpoint for imipenem susceptibility (≤1 μg/mL).[16]

Checkerboard assays to determine whether compound **3d** is acting synergistically (ΣFIC ≤ 0.5) with carbapenem antibiotics were performed as described previously.[17] The ΣFIC calculated for compound **3d** with both imipenem and meropenem was 0.375, indicating that the combinations are synergistic.

We then further validated the adjuvant activity of the lead compound **3d** by constructing time−kill curves from colony-forming unit (CFU) data at time points up to 16 h (Figure 2). This serves as another method to confirm that the combination of compound **3d** and the carbapenem is acting synergistically (≥2 $\log_{10}$ decrease in the number of CFUs relative to the count obtained with the antibiotic alone indicates synergy), in addition to allowing us to monitor the effect of compound

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3d as a function of time. *K. pneumoniae* cultures were grown in various concentrations of imipenem in the presence and absence of compound **3d** (30 μM) in CAMHB. Bacteria were plated at various time points, and the number of viable CFUs was enumerated. The reduction in CFUs as compared to control bacteria is displayed in Table 2.

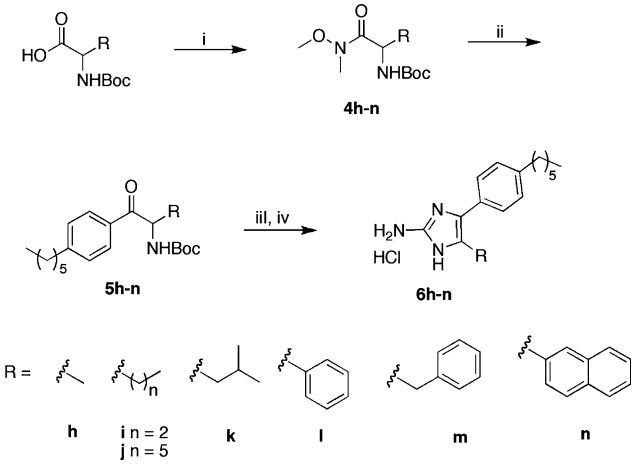
Table 2. Log Reduction in *K. pneumoniae* CFU by Imipenem in the Presence and Absence of Compound 3d

		Log reduction in CFU/mL as compared to untreated control	
time (h)	imipenem concn (μg/mL)	imipenem alone	imipenem + 30 μM **3d**
	32	4.36 ± 0.06	>5.0
	16	3.16 ± 0.05	>5.0
4	8	2.53 ± 0.12	>5.0
	4	0.86 ± 0.06	>5.0
	2	0.79 ± 0.02	3.84 ± 0.12
	32	0.92 ± 0.03	>5.0
	16	0.39 ± 0.05	>5.0
8	8	0.62 ± 0.07	>5.0
	4	0.53 ± 0.01	3.71 ± 0.20
	2	0.38 ± 0.03	0.88 ± 0.17
	32	<0.1	>5.0
	16	<0.1	>5.0
16	8	<0.1	2.40 ± 0.19
	4	<0.1	0.30 ± 0.08
	2	<0.1	<0.1

As can be seen, we observe more marked reduction in CFUs for combination treatments as compared to treatment with imipenem alone. The effect of compound **3d**, when compared to imipenem alone, is most apparent from 4 h of incubation onward (after bacteria leave lag phase); for example, at an imipenem concentration of 16 μg/mL (1/4 MIC), after 4 h, the antibiotic alone reduces the number of CFUs by only 3.16 log units in comparison to untreated control, while the addition of compound **3d** results in a >5.0 log unit reduction. After 8 h, this effect is even more marked with only a 0.39 log unit reduction for the antibiotic alone as compared to a >5.0 log unit reduction in the presence of compound **3d**. In addition, a considerable reduction in the number of CFUs can still be observed at lower imipenem concentrations; for example, at 4 μg/mL (1/16 MIC), the addition of compound **3d** results in a >5.0 log reduction, as compared to just 0.86 log reduction for imipenem alone after 4 h of incubation. The growth of bacteria cultured in the presence of imipenem alone recovers very quickly (after 2 h), possibly due to degradation of the antibiotic by the NDM-1 producing bacteria. The addition of compound **3d** slows this recovery, although a rebound in growth is still observed for combination treatments at all imipenem concentrations lower than 32 μg/mL. Regrowth, however, is typical of bacterial cultures (both resistant and sensitive) treated with β-lactam antibiotics.

In an effort to improve the efficacy of compound **3d** and to explore the effects of further substitution of the 2-AI heterocycle, we synthesized a second generation of 2-AI-aryl analogues that possessed a 4,5-disubstitution pattern. We have previously observed that the introduction of substituents at the 5-position of compound **1** resulted in increased suppression of oxacillin resistance in MRSA.[17] Substituents were chosen to allow comparison of the effect of straight chain alkyl groups of varying lengths, branched alkyl groups, and aromatic groups of varying size. The synthetic approach to these second-generation aryl 2-AI analogues is outlined in Scheme 2. A variety of readily

Scheme 2. Synthesis of 4,5-Disubstituted Analogues of Compound 3d[a]



[a]Reagents and conditions: (i) HN(OMe)Me·HCl, BOP, Et_3N, CH_2Cl_2, room temperature, 16 h. (ii) 4-Hexylphenyl magnesium bromide, 2-methyltetrahydrofuran (0.5 M), −20 °C−room temperature, 16 h. (iii) TFA, CH_2Cl_2, room temperature, 2 h. (iv) EtOH/H_2O, H_2NCN, pH 4.3, 95 °C, 3 h.

available Boc-protected amino acids with aromatic and straight chain and branched alkyl substituents were used as the starting point for this synthesis.

Conversion of the starting carboxylic acids to the corresponding Weinreb amides **4h−n** was followed by addition of 4-hexylphenyl magnesium bromide to form a series of α-amino ketones **5h−n**. Amine deprotection and subsequent condensation with cyanamide afforded 4,5-disubstituted 2-AIs **6h−n**. After purification, each compound was converted to the corresponding HCl salt for biological testing.

The second-generation compounds were then tested for their ability to lower the imipenem and meropenem MICs of the NDM-1 producing strain of *K. pneumoniae* (Table 3).

Table 3. NDM-1 *K. pneumoniae* Carbapenem MICs in the Presence of Compounds 6h−n

	MIC (μg/mL)	
compd (MIC/concn tested)	imipenem	meropenem
no compd	64	256
6h (100/30 μM)	8	32
6i (100/30 μM)	16	64
6j (>200/50 μM)	64	128
6k (200/50 μM)	32	64
6l (>200/50 μM)	32	128
6m (>200/50 μM)	32	128
6n (>200/50 μM)	64	128

Unfortunately, however, unlike our previous MRSA study, we found that introduction of substituents at the 5-position of compound **3d** did not result in increased activity; nonetheless, compounds **6h** and **6i**, in which the 5-position substituent is a methyl and propyl group, respectively, were able to lower MIC values. Compound **6h** effected an 8-fold drop in the MICs of

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both imipenem and meropenem at a concentration of 30 μM. Increasing the chain length of the new substituent to six carbons resulted in almost complete loss of activity, as did the introduction of branched (iso-butyl) and aromatic (phenyl, benzyl, and naphthyl) groups.

We next determined whether the resistance suppression activity of compound **3d** was specific to the NDM-1 producing strain of *K. pneumoniae* or if compound **3d** was able to suppress carbapenem resistance in other MDR *K. pneumoniae*. To address this issue, we investigated the ability of compound **3d** to suppress resistance in two other β-lactam-resistant strains of *K. pneumoniae*. Against the KPC-2 producing strain (ATCC BAA-1705) at 30 μM, compound **3d** was able to reduce the imipenem MIC by 16-fold (from 32 to 2 μg/mL), and against an ESBL producing strain (ATCC 700603), compound **3d**, at 30 μM, reduced the cefotaxime MIC by 8-fold (from 8 to 1 μg/mL).

Finally, preliminary investigations into the mechanism of action of the lead compound were carried out. Given the amphiphilic nature of the lead compound **3d**, we examined the effect that it has on the permeability of the *K. pneumoniae* cell membrane. Bacterial membrane damage was examined using the Baclight assay as described by Hilliard et al.[18] After exposure to compound **3d** for 1 h, the permeability of the *K. pneumoniae* membrane (NDM-1 producing strain) was considerably increased, with an intact/permeabilized membrane ratio of 8.5% of the control (DMSO only treated) bacteria at its active resistance suppression concentration of 30 μM. To investigate whether this is the sole mechanism by which compound **3d** is able to suppress antibiotic resistance, we also examined the effect of two compounds that were much less active as suppressors of carbapenem resistance than compound **3d**. Compound **3e**, which differs from compound **3d** only by one extra methylene unit in the tail, and effected a reduction in carbapenem MIC of just 2−4-fold, resulted in an intact/permeabilized membrane ratio of 21.2% of the control bacteria at a concentration of 50 μM. From the second generation of analogues, compound **6j**, which possesses a hexyl chain at the 4-position of the 2-AI ring and did not reduce the imipenem MIC at all (reduction of meropenem MIC was 2-fold), had a comparable effect on membrane permeability to compound **3d**, with an intact/permeabilized membrane ratio of 8.13% of the control bacteria at a concentration of 30 μM. The fact that two much less active compounds affected bacterial membrane permeability considerably suggests that this is not the sole mechanism by which compound **3d** is able to lower antibiotic resistance.

Because of the effect that compound **3d** has on the bacterial membrane, we were interested in the effect that the compound has on eukaryotic cell membranes, as an indicator of the potential of this scaffold to be used as an antibiotic adjuvant. We therefore investigated the hemolytic activity of compound **3d** using mechanically difibrinated sheep blood as described previously.[15] Compound **3d** had a much smaller effect on blood cell membranes as compared to bacterial membranes. The HD_{50} (the concentration at which the compound lyses 50% cells as compared to the positive control) of compound **3d** was >500 μM, and the % lysis at the active concentration (30 μM) was 2.5%.

In conclusion, we have identified a series of 2-AI aryl compounds that have the ability to suppress carbapenem resistance in a NDM-1 producing strain of *K. pneumoniae*, with some compounds able to lower the MIC of both imipenem and meropenem by 8−16-fold at a concentration (30 μM) at which the lead compound itself displays little microbicidal activity. Assaying at a slightly elevated concentration (50 μM) resulted in a 256-fold reduction in imipenem MIC. Furthermore, the lead compound of this series is also able to suppress resistance in other carbapenem- and cephalosporin-resistant *K. pneumoniae* strains. The lead compound, along with two much less active compounds, was shown to considerably affect permeabilization of the *K. pneumoniae* cell membrane. The lead compound was shown to have very little hemolytic activity at the concentration at which it affected the bacterial membrane. Studies are currently underway to determine the mechanism of action of these compounds and to identify the molecular targets, along with further analogue synthesis to identify more efficacious compounds. Given the urgent need for new strategies to deal with the problem of MDR Gram-negative bacteria, the identification of a novel small molecule that is able to effect such a marked suppression of carbapenem resistance represents an opportunity not only for the development of new therapeutic entities but also for the identification of new targets for future medicinal chemistry efforts.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic methods and compound characterization for all new compounds and protocols for MIC determination and time−kill curves, along with representative ^{1}H NMR and ^{13}C NMR spectra and membrane permeability assays. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ccmeland@ncsu.edu.

Author Contributions

R.J.W., C.A.B., and C.S.R. performed the experiments outlined in the manuscript, while R.J.W., C.A.B., and C.M. contributed to writing the manuscript.

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Notes

The authors declare the following competing financial interest(s):CM has significant financial interest in a corporate entity seeking to commercialize 2-AI derivatives as antibiotic adjuvants.

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