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Exploration of a benzothiophene scaffold for use as adjuvants with β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus*

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Rising antibiotic resistance coupled with diminishing investment in antibiotic development has led to limited treatment options for multi drug-resistant bacterial infections. Penicillin binding protein 2a (PBP2a) is a key determinant of resistance to many β-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* (MRSA), while over-expression of PBP4 mediates resistance to fifth-generation cephalosporins. We previously identified benzothiophene **NDM-335** as an inhibitor of PBP4 that overcomes PBP4 mediated resistance to ceftobiprole, and have shown that other PBP4 inhibitor scaffolds can be tuned to overcome PBP2a-mediated resistance. Based upon this precedent, we conducted a structure activity relationship (SAR) study on the **NDM-335** scaffold to uncover compounds that overcome PBP2a-mediated oxacillin resistance in MRSA, with the lead compound lowering the minimum inhibitory concentration 32-fold at 5 μM. Further development of this new adjuvant lead could potentially deliver a new combination therapy for treating MRSA infections.

1. Introduction

Antibiotic resistance is a well-established global health threat that was exacerbated during the coronavirus disease pandemic in 2019 (COVID-19).[1] The Centers for Disease Control and Prevention (CDC) reported that almost 30 000 people died from antimicrobial resistant bacterial infections during the pandemic's first year, with 40% of these deaths caused by nosocomial infections.[1] *Staphylococcus aureus* is one of the six ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species), a group of bacterial pathogens that are frequently associated with hospital associated infections, and are notorious for their multidrug resistance.[2] The CDC reported in July 2024 that infections caused by ESKAPE pathogens increased by a combined 20% during the pandemic compared to the pre-pandemic period.[1,3] Although the rate of methicillin resistant *S. aureus* (MRSA) infections returned to pre-pandemic levels in 2022, continued stewardship and antibiotic development are required to control MRSA infections.[3]

The introduction of new antibiotics has historically been employed to combat multidrug resistant (MDR) bacterial threats; however, the rapid emergence of resistance, coupled with large pharmaceutical companies' exit from antibiotic development has prompted orthogonal approaches to address this problem.[4] One solution is the use of adjuvants, which are compounds that do not possess standalone antimicrobial properties, but potentiate the activity of partner antibiotics by circumventing bacterial resistance mechanisms.[5] The most well-known, and only clinically approved adjuvants to date, are serine β-lactamase inhibitors, such as clavulanic acid which is paired with amoxicillin and marketed as Augmentin.[6] In addition to Augmentin, avibactam is a non-β-lactam serine β-lactamase inhibitor that was clinically approved in 2015 and is administered in combination with ceftazidime.[7] Outside of β-lactamase inhibitors, many other resistance mechanisms represent potential targets for the adjuvant approach.[8]

Penicillin binding proteins (PBPs) are transpeptidases/transglycosylases that play a role in the formation of the peptidoglycan component of the bacterial cell wall. *S. aureus* encodes four native PBPs; two essential (PBP1 and PBP2) and two non-essential (PBP3 and PBP4).[9–11] While resistance to β-lactams in MRSA predominantly occurs through the non-native PBP2a (encoded by *mecA*), which has a lower affinity for most β-lactams than do native PBPs, PBP2a-independent β-lactam resistance has also been reported.[12–14] This latter resistance is derived from the overexpression of PBP4, which confers resistance to the fifth-generation cephalosporins,

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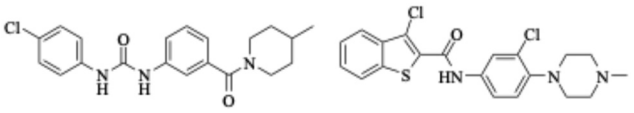


Fig. 1 Structure of 9314848 (left) and **NDM-335** (right).

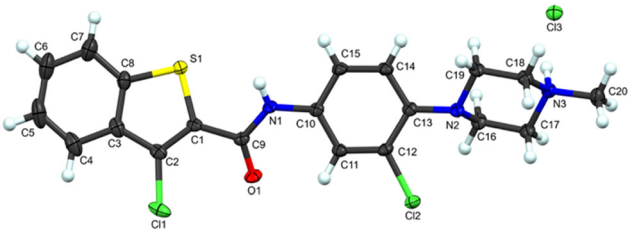


Fig. 2 Crystal structure of **NDM-335**.

ceftobiprole (BPR) and ceftaroline, which are otherwise active against MRSA.[15] This discovery prompted us to screen for potentiators of BPR in a PBP4 overexpressing strain of *S. aureus* to identify potential inhibitors of PBP4.[16] One hit from this screen was the phenyl urea ChemBridge 9314848 (Fig. 1), which inhibits binding of a fluorescently labeled penicillin molecule (Bocillin-FL) to PBP4 and, interestingly, PBP2a.[17] Compound 9314848 also potentiates β-lactams against MRSA strains with PBP2a-derived resistance, and analog synthesis led to the identification of compounds with increased activity with select penicillins and cephalosporins.[18]

Another compound identified from the screen for PBP4 inhibitors was **NDM-335** (ChemBridge 7974147) (Fig. 1).[16] At 25 μM, **NDM-335** lowers the minimum inhibitory concentration (MIC) of BPR to 8 μg mL^{-1} against the BPR-resistant, PBP4 over-expressor CRB without affecting expression of *pbp4*.[16] CRB is a derivative of the MRSA strain COL that lacks the *mecA* gene coding for PBP2a, and over-expresses PBP4 leading to broad resistance to β-lactams, including fifth generation cephalosporins.[13] Given that ChemBridge 9314848 could be tuned to reduce β-lactam resistance in PBP2a-expressing strains with normal PBP4 expression levels, we elected to further explore the potential of **NDM-335** and analogs as antibiotic adjuvants targeting PBP2a-dependent resistance.

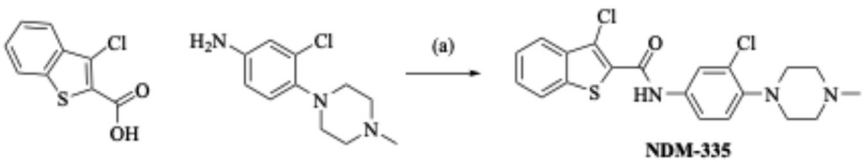
2. Results and discussion

We first resynthesized **NDM-335** *via* a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride mediated coupling of commercially available 3-chlorobenzothiophene-2-carboxylic acid and 3-chloro-4-(4-methylpiperazin-1-yl)aniline (Scheme 1).[19] We further verified the structure of **NDM-335** by solving its crystal structure (Fig. 2).

We first determined the standalone MIC of **NDM-335** against the MRSA USA300 strain BAA-1556 (PBP2a/normal PBP4 expression)[20] under standard microdilution assay conditions to be >200 μM. We began a series of oxacillin potentiation assays, which are commonly used to identify other inhibitors of PBP2a related pathways.[21,22] At 60 μM (<30% standalone MIC), **NDM-335** lowers the oxacillin MIC against this strain from 32 μg mL^{-1} to 0.125 μg mL^{-1} (256-fold). Against MRSA, the Clinical Laboratory Standards Institute (CLSI) breakpoint for oxacillin is 2 μg mL^{-1}.[23] We sequentially performed a dose–response study to determine the lowest concentration of **NDM-335** that lowers the oxacillin MIC to or below the clinical breakpoint, and term this the adjuvant's minimum effective concentration (MEC). Against BAA-1556, at 7.5 μM, **NDM-335** lowers the oxacillin MIC to 0.25 μg mL^{-1}, while at 3.75 μM, it suppresses the MIC to 8 μg mL^{-1} (Table 1), indicating the MEC lies between 7.5 and 3.75 μM. We then evaluated **NDM-335** against two additional MRSA strains: ATCC 43300 and AH-1263 (standalone oxacillin MICs of 32 μg mL^{-1} for each strain), both of which also harbor the *mecA* gene and show normal PBP4 expression levels. **NDM-335** lowers the oxacillin MIC to 0.5 μg mL^{-1} at 5 μM in ATCC 43300, and to 0.25 μg mL^{-1} at 7.5 μM in AH-1263. As a control, we measured potentiation of BPR by **NDM-335** against BAA-1556. In this strain, the BPR MIC is 1 μg mL^{-1}, and this is only reduced four-fold in the presence of 12.5 μM (and concentrations up to 200 μM) **NDM-335**, indicating strong specificity for PBP2a-dependent resistance.

With these promising results, we initiated a structure activity relationship (SAR) study of the benzothiophene region of **NDM-335** to determine whether compounds with enhanced adjuvant activity could be identified. To this end, we synthesized a library of compounds in which the benzothiophene was replaced with alternative heterocycles (or a naphthyl ring), while conserving the 3-chloro-4-(4-methylpiperazine) moiety. Following the synthetic route outlined in Scheme 1, the indole, 3-chloroindole, benzothiazole, furan, benzofuran, and naphthalene derivatives (Fig. 3) were synthesized from the respective commercially available carboxylic acids. Indole, 3-chloroindole, benzothiazole, benzofuran, and naphthalene were selected for their structural similarities to benzothiophene, and to analyze the impact of altering the heteroatom of the benzothiophene head group. The furan was chosen to assess the necessity of the benzyl ring and the sulfur atom.

The 3-chlorobenzofuran (**NDM-713**) analog required a more complex synthesis (Scheme 2), starting with subjecting benzofuran-3(2*H*)-one to Vilsmeier–Haack conditions[24] to



Scheme 1 (a) EDC, DMAP, DCM, rt, 24 h, 49.5% yield.

Table 1 Oxacillin MICs against MRSA ATCC BAA-1556, MRSA 43300, and MRSA AH-1263 in the presence and absence of **NDM-335**

NDM-335 standalone MIC (μM)	**NDM-335** concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556		
>200	—	**32**
	60	0.125 [256]
	30	0.125 [256]
	15	0.125 [256]
	7.5	0.25 [128]
	3.75	8 [4]
MRSA ATCC 43300		
>200	—	**32**
	60	0.125 [256]
	30	0.125 [256]
	15	0.125 [256]
	7.5	0.125 [256]
	5	0.5 [64]
	4	4 [8]
MRSA AH-1263		
>200	—	**32**
	60	0.125 [256]
	30	0.25 [128]
	15	0.25 [128]
	7.5	0.25 [128]
	5	8 [4]

provide 3-chlorobenzofuran-2-carbonitrile, **1**. The nitrile was then treated with sodium hydroxide to provide key intermediate **2**, which was then reacted with 3-chloro-4-(4-methylpiperazin-1-yl)aniline *via* an EDC-mediated coupling to yield **NDM-713.** To access the 2-chloronapthalene (**NDM-712**) derivative (Scheme 2), 3-amino-2-naphthoic acid was subjected to Sandmeyer conditions to provide 3-chloro-2-naphthoic acid, 3.[25] 3-Chloro-2-naphthoic acid was then reacted with 3-chloro-4-(4-methylpiperazin-1-yl)aniline *via* an EDC coupling to provide amide **NDM-712.**

The stand-alone MIC of each compound was first determined against the same three MRSA strains described above (Table 2), with all compounds, except the 3-chloroindole derivative **NDM-704**, returning MICs of 100 to >200 μM. Due to low solubility of **NDM-704** in stock solution of DMSO, it was tested at ten-fold lower starting concentrations and returned a stand-alone MIC greater than 20 μM in BAA-1556, 43300, and AH-1263. Oxacillin MICs in the presence of each analog are summarized in Table 2. The naphthyl (**NDM-365**), indole (**NDM-406**), and furan (**NDM-407**) derivatives fail to potentiate oxacillin against BAA-1556 and AH-1263, returning unchanged oxacillin MICs of 32 μg mL^{-1} at 60 μM. Against 43300, **NDM-365** and **NDM-407** exhibit minimal activity, lowering the oxacillin MIC only four-fold to 8 μg mL^{-1} at 60 μM. Against this same strain, **NDM-406** lowers the oxacillin MIC 32-fold to 1 μg mL^{-1} at 60 μM, and 64-fold to 0.5 μg mL^{-1} at 30 μM, but lost significant activity at 15 μM with an oxacillin MIC of 4 μg mL^{-1} (eight-fold reduction). Against BAA-1556, the benzofuran derivative (**NDM-511**) lowers the oxacillin MIC 32-fold to 1 μg mL^{-1} at 60 μM,

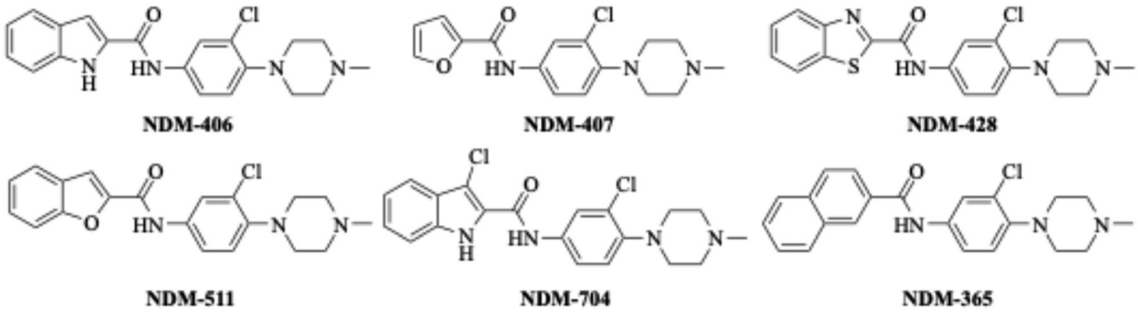
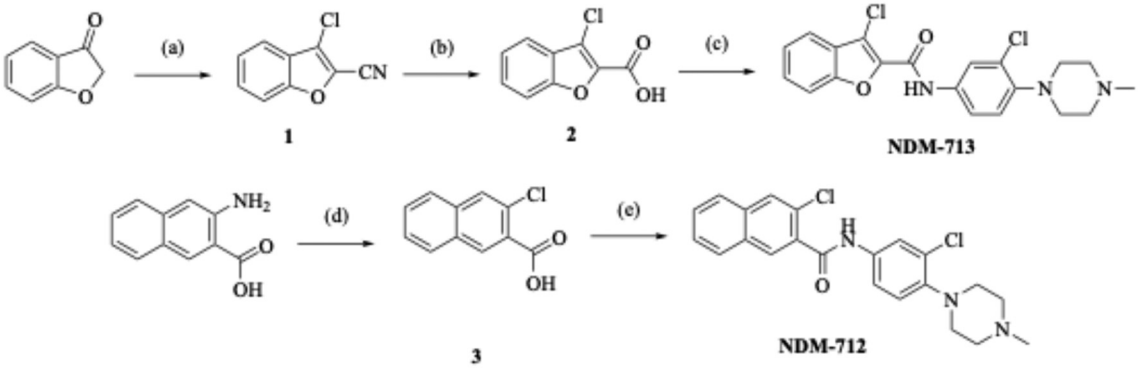


Fig. 3 Benzothiophene heterocycle substitutions.



Scheme 2 (a) $POCl_3$, DMF 0 °C – rt 1 h, NH_2OH 0 °C – rt, 1 h, quant. yield. (b) NaOH, EtOH : H_2O, 100 °C, 2 h, 14% yield. (c) EDC, DMAP, DCM, rt, 24 h. (d) 3 M HCl, $NaNO_2$, CuCl 0 °C, 1 h, 17% yield. (e) EDC, DMAP, DCM, rt, 24 h, 31% yield.

Table 2 Oxacillin MICs against MRSA ATCC BAA-1556, MRSA ATCC 43300, and MRSA AH-1263 in the presence of benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556			
—	—	—	**32**
NDM-365	>200	60	32 [–]
NDM-406	>200	60	32 [–]
NDM-407	>200	60	32 [–]
NDM-428	>200	60	2 [16]
		30	1 [32]
		15	1 [32]
		7.5	1 [32]
		5	2 [16]
		4	4 [8]
NDM-511	>200	60	1 [32]
		30	8 [4]
NDM-704	>20	6	16 [2]
NDM-712	>200	60	16 [2]
NDM-713	100	30	0.125 [256]
		15	32 [–]
MRSA ATCC 43300			
—	—	—	**32**
NDM-365	>200	60	8 [4]
NDM-406	>200	60	1 [32]
		30	0.5 [64]
		15	4 [8]
NDM-407	>200	60	8 [4]
NDM-428	>200	60	16 [2]
NDM-511	200	60	0.25 [128]
		30	4 [8]
NDM-712	>200	60	8 [4]
NDM-713	100	30	0.125 [256]
		15	16 [2]
NDM-704	>20	6	16 [2]
MRSA AH-1263			
—	—	—	**32**
NDM-365	>200	60	32 [–]
NDM-406	>200	60	32 [–]
NDM-407	>200	60	32 [–]
NDM-428	>200	60	0.5 [64]
		30	0.5 [64]
		15	0.25 [128]
		7.5	0.5 [64]
		5	8 [4]
NDM-511	>200	60	0.5 [64]
		30	8 [4]
NDM-712	>200	60	2 [16]
		30	4 [8]
NDM-713	100	30	0.25 [128]
		15	16 [2]
NDM-704	>20	6	4 [8]

and four-fold (8 μg mL^{-1}) at 30 μM. Against 43300, **NDM-511** returns an oxacillin MIC of 0.25 μg mL^{-1} at 60 μM, which increases to 4 μg mL^{-1} at 30 μM. Against AH-1263, **NDM-511** suppresses the oxacillin MIC to 0.5 μg mL^{-1} at 60 μM, which increases to 8 μg mL^{-1} at 30 μM. Against BAA-1556 and 43300, **NDM-704** is inactive at 6 μM, returning oxacillin MICs of 16 μg mL^{-1}, an insignificant two-fold drop from the standalone oxacillin MIC. In AH-1263, **NDM-704** delivers an eight-fold reduction in oxacillin MIC to 4 μg mL^{-1} at 6 μM. The 2-chloronaphthyl derivative (**NDM-712**) lowers the oxacillin MIC to 16 μg mL^{-1} and 8 μg mL^{-1} at 60 μM, in BAA-1556 and 43300,

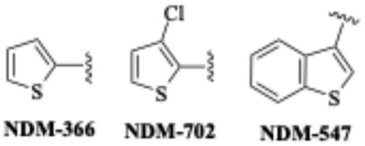


Fig. 4 Sulfur-containing heterocycle analogs.

respectively. **NDM-712** reduces the oxacillin MIC to the breakpoint of 2 μg mL^{-1} at 60 μM in AH-1263. and to 4 μg mL^{-1} at 30 μM. The 3-chlorobenzofuran derivative (**NDM-713**) suppresses the oxacillin MIC to 0.125 μg mL^{-1}, 0.125 μg mL^{-1}, and 0.25 μg mL^{-1} against BAA-1556, 43300, and AH-1263, respectively, at 30 μM. At 15 μM, the oxacillin MIC increases to 32 μg mL^{-1}, 16 μg mL^{-1}, and 16 μg mL^{-1} against these three strains. Finally, the benzothiazole derivative (**NDM-428**) lowers the MIC of oxacillin below the breakpoint at 5 μM in BAA-1556 (to 2 μg mL^{-1}) and at 7.5 μM in AH-1263 (to 0.5 μg mL^{-1}) but interestingly is not active in 43300 (returning an oxacillin MIC of 16 μg mL^{-1}). From this, it appears that the presence of a sulfur atom at the one-position of the heterocycle is important for oxacillin adjuvant activity.

Based upon the importance of the sulfur atom, we next investigated compounds with various sulfur containing heterocycles (Fig. 4). Each derivative was synthesized from the respective carboxylic acid using the approach outlined in Scheme 1. Analogs synthesized include: **NDM-366** that contains a thiophene, **NDM-702** with a 3-chlorothiophene, and **NDM-547** that harbors a benzothiophene group attached at the 3-position, rather than the 2-position as seen in **NDM-335**.

The thiophene derivatives **NDM-366** and **NDM-702** have standalone MICs greater than 200 μM against all three strains, while **NDM-547** exhibits a standalone MIC of 100 μM against each strain (Table 3). **NDM-702** is inactive at 60 μM, returning oxacillin MICs of 16 μg mL^{-1} against all three strains. **NDM-366** is inactive against BAA-1556, and AH-1263, and only lowers the oxacillin MIC four-fold to 8 μg mL^{-1} against 43300 at 60 μM. **NDM-547** returns an oxacillin MIC of 0.5 μg mL^{-1} at 30 μM in BAA-1556, but loses activity at lower concentrations, only reducing the oxacillin MIC to 4 μg mL^{-1} at 15 μM. Against 43300, **NDM-547** lowers the oxacillin MIC to 1 μg mL^{-1} at 15 μM, but fails to reach breakpoint levels at 10 μM, returning an MIC of 4 μg mL^{-1}. In AH-1263, **NDM-547** lowers the MIC of oxacillin to 0.5 μg mL^{-1} at 30 μM, and to 16 μg mL^{-1} at 15 μM. In aggregate, these data underscore the importance of a benzothiophene motif for activity, with connection at the C-2 position delivering the highest activity. To follow up on this observation, additional C-2 substituted benzothiophenes were synthesized (Fig. 5) again *via* the route shown in Scheme 1. These include: the unsubstituted analog (**NDM-400**), 5-methylbenzothiophene (**NDM-427**), 5-chlorobenzothiophene (**NDM-419**), 6-chlorobenzothiophene (**NDM-703**), 3-methylbenzothiophene (**NDM-343**), and 6-methylbenzothiophene (**NDM-408**).

Standalone MIC and adjuvant activity for these analogs is summarized in Table 4. The 5-chlorobenzothiophene analog **NDM-419** is inactive at 60 μM against BAA-1556 and AH-1263,

Table 3 Oxacillin MICs against MRSA ATCC BAA-1556, MRSA ATCC 43300, and MRSA AH-1263 in the presence of benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556			
—	—	—	**32**
NDM-366	>200	60	32 [–]
NDM-547	100	30	0.5 [64]
		15	4 [8]
NDM-702	>200	60	16 [2]
MRSA ATCC 43300			
—	—	—	**32**
NDM-366	>200	60	8 [4]
NDM-547	100	30	0.5 [64]
		15	1 [32]
		10	4 [8]
		7.5	4 [8]
NDM-702	>200	60	16 [2]
MRSA AH-1263			
—	—	—	**32**
NDM-366	>200	60	32 [–]
NDM-547	100	30	0.5 [64]
		15	16 [2]
NDM-702	>200	60	16 [2]

while it is only moderately active against 43300, returning oxacillin MICs of 32 μg mL^{-1}, 16 μg mL^{-1}, and 8 μg mL^{-1}, respectively. The 6-chloro derivative **NDM-703** exhibits a similar lack of activity, returning oxacillin MICs of 16 μg mL^{-1}, 8 μg mL^{-1}, and 16 μg mL^{-1} against BAA-1556, 43300, and AH-1263, respectively at 60 μM. The 6-methyl derivative **NDM-408** is moderately active against BAA-1556 and AH-1263, lowering the oxacillin MIC four-fold to 8 μg mL^{-1} at 60 μM; however, it exhibits greater activity against 43300, lowering the oxacillin MIC to 2 μg mL^{-1} at 5 μM. The unsubstituted derivative **NDM-400** lowers the oxacillin MIC to 1 μg mL^{-1} at 7.5 μM and 2 μg mL^{-1} at 5 μM in BAA-1556 and 43300, respectively, and to 0.5 μg mL^{-1} at 15 μM against AH-1263. The 5-methyl derivative **NDM-427** retains activity at 7.5 μM against all three strains, lowering the oxacillin MIC to 2 μg mL^{-1}. As a result of the initial SAR study, the parent **NDM-335**, the unsubstituted benzothiophene **NDM-400**, the 6-methylbenzothiophene **NDM-408**, the 5-methylbenzothiophene **NDM-427**, and the benzothiazole **NDM-428** were subject to further investigation. **NDM-335**, **NDM-408**, **NDM-427**, and **NDM-428** were chosen as lead compounds because they potentiate oxacillin at concentrations of 10 μM or lower in at least two out of the three strains tested. While **NDM-400** does not achieve this same standard, we chose it as a lead compound due to its activity at 5 μM in MRSA 43300 and 15 μM in MRSA AH-1263.

Lead compounds were tested against the methicillin sensitive *S. aureus* (MSSA) strain ATCC 29213 as a control (Table 5). MSSA 29213 lacks PBP2a, leading us to predict that potentiation activity would be lost. **NDM-400**, **NDM-408**, **NDM-427**, and **NDM-428** lower the MIC of oxacillin to 0.125 μg mL^{-1} at 60 μM, which is two-fold lower than the standalone oxacillin MIC of 0.25 μg mL^{-1} in MSSA 29213. MIC changes of two fold are considered statistically insignificant, thus strongly indicating that the potentiation activity is related to PBP2a, or other regulatory networks involved in PBP2a-mediated resistance.

Following the study of benzothiophene derivatives in combination with oxacillin, activity of lead compounds with two additional β-lactams: penicillin G (Table 6), and the second-generation cephalosporin, cefoxitin (Table 7) was explored. In BAA-1556 and MRSA 43300, three of the five compounds (**NDM-408**, **NDM-427**, and **NDM-428**) did not reduce the MIC of penicillin G to its clinical breakpoint of 0.125 μg mL^{-1} at 60 μM (Table 7).[23] **NDM-335** lowers the penicillin G MIC to 0.0625 at 60 μM, 30 μM, and 15 μM, but fails to achieve breakpoint levels at 10 μM. **NDM-400** returns a penicillin G MIC of 0.015625 μg mL^{-1} at 60 μM and 0.125 μg mL^{-1} at 30 μM but fails to achieve breakpoint levels at 15 μM. In MRSA 43300, **NDM-335**, **NDM-408**, **NDM-427**, and **NDM-428** do not achieve breakpoint level MICs when dosed at 60 μM. **NDM-400** lowers the penicillin G MIC to 0.03125 μg mL^{-1} at 60 μM, but the MIC increases to 0.5 μg mL^{-1} when dosed at 30 μM. Against MRSA AH-1263, **NDM-408**, **NDM-427**, and **NDM-428** return MICs greater than the penicillin G breakpoint of 0.125 μg mL^{-1} at 60 μM. **NDM-335** and **NDM-400** return penicillin G MICs of 0.125 μg mL^{-1} and 0.015625 μg mL^{-1} respectively at 60 μM. Both compounds return penicillin MICs of 0.125 μg mL^{-1} at 30 μM, but activity is lost at 15 μM. At 15 μM, **NDM-335** and **NDM-400** return penicillin G MICs of 4 μg mL^{-1}.

Lead compounds were tested with cefoxitin. Against MRSA BAA-1556, **NDM-335**, **NDM-408**, **NDM-427**, and **NDM-428** fail to lower the cefoxitin MIC to its clinical breakpoint level of 4 μg mL^{-1}.[23] **NDM-335** lowers the cefoxitin MIC to 0.125 μg mL^{-1} at 60 μM, and 16 μg mL^{-1} at 30 μM. Against MRSA 43300, **NDM-335**, **NDM-408**, **NDM-427**, and **NDM-428** fail to lower the cefoxitin MIC to breakpoint levels. **NDM-400** lowers the cefoxitin MIC to 0.5 μg mL^{-1} at 60 μM and 16 μg mL^{-1} at 30 μM, showing a loss of activity at 30 μM. In MRSA AH-1263, **NDM-400**, **NDM-408**, **NDM-427**, and **NDM-428** fail to lower the cefoxitin MIC to breakpoint levels. Taken together, these results are similar to other adjuvants we have developed, where there appears to be an optimal antibiotic partner within the general antibiotic class.[18,26,27]

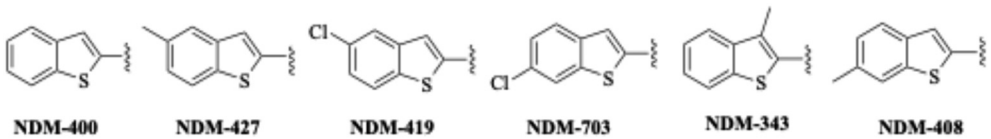


Fig. 5 Substituted benzothiophene analogs.

Table 4 Oxacillin MICs against MRSA ATCC BAA-1556, MRSA ATCC 43300, and MRSA AH-1263 in the presence of benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556			
—	—	—	**32**
NDM-408	>200	60	8 [4]
NDM-343	>200	60	2 [16]
		30	2 [16]
		15	4 [8]
NDM-400	>200	60	0.125 [256]
		30	0.125 [256]
		15	0.25 [128]
		7.5	1 [32]
		5	16 [2]
NDM-419	>200	60	32 [–]
NDM-427	>200	60	0.25 [128]
		30	0.5 [64]
		15	1 [32]
		7.5	2 [16]
		5	8 [4]
NDM-703	>200	60	16 [2]
MRSA ATCC 43300			
—	—	—	**32**
NDM-343	>200	60	0.25 [128]
		30	2 [16]
		15	4 [8]
NDM-400	>200	60	0.125 [256]
		30	0.125 [256]
		15	0.5 [64]
		7.5	2 [16]
		5	2 [16]
		4	4 [8]
NDM-408	>200	60	0.25 [256]
		30	0.25 [256]
		15	0.25 [256]
		7.5	0.5 [64]
		5	2 [16]
		4	4 [8]
NDM-419	>200	60	8 [4]
NDM-427	>200	60	0.125 [256]
		30	0.125 [256]
		15	0.125 [256]
		7.5	2 [16]
		5	4 [8]
NDM-703	>200	60	8 [4]
MRSA AH-1263			
—	—	—	**32**
NDM-343	>200	60	16 [2]
NDM-400	>200	60	0.125 [256]
		30	0.25 [128]
		15	0.5 [64]
		10	16 [2]
NDM-408	>200	60	8 [4]
NDM-419	>200	60	16 [2]
NDM-427	>200	60	0.125 [256]
		30	0.5 [64]
		15	0.5 [64]
		7.5	2 [16]
		5	8 [4]
NDM-703	>200	60	16 [2]

Growth curves were then constructed for **NDM-335**, **NDM-400**, and **NDM-427** using MRSA ATCC 43300 as the test strain (Fig. 6–8). Each compound at its MEC in combination with oxacillin at the breakpoint concentration (2 μg mL^{-1}) was examined, along with 2× and 4× this dosing. Based on the changes in colony forming units (CFUs), the combination of **NDM-335**, **NDM-400**, and **NDM-427** with oxacillin was determined to be bacteriostatic.[28]

S. aureus codes for a repertoire of two component regulatory systems (TCS), including VraRS, ArlRS, WalKR, BlaIR, GraRS, SaeRS, that have been shown to directly or indirectly modulate resistance to cell wall active antibiotics.[29] Thus, to begin a preliminary investigation into the mechanism of action, lead compounds were examined against a panel of deletion strains

Table 5 Oxacillin MICs against MSSA 29213 in the presence of lead benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
MSSA ATCC 29213			
—	—	—	**0.25**
NDM-335	>200	60	0.125 [2]
NDM-400	>200	60	0.125 [2]
NDM-408	>200	60	0.125 [2]
NDM-427	>200	60	0.125 [2]
NDM-428	>200	60	0.125 [2]

Table 6 Penicillin G MICs against MRSA ATCC BAA-1556, MRSA ATCC 43300, and MRSA AH-1263 in the presence of benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Penicillin G MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556			
—	—	—	**4**
NDM-335	>200	60	0.0625 [64]
		30	0.0625 [64]
		15	0.0625 [64]
		10	0.5 [8]
NDM-400	>200	60	0.015625 [256]
		30	0.125 [32]
		15	0.25 [16]
NDM-408	>200	60	2 [2]
NDM-427	>200	60	0.25 [16]
NDM-428	>200	60	2 [2]
MRSA ATCC 43300			
—	—	—	**4**
NDM-335	>200	60	0.25 [16]
NDM-400	>200	60	0.03125 [128]
		30	0.5 [8]
NDM-408	>200	60	8 [–]
NDM-427	>200	60	1 [4]
NDM-428	>200	60	8 [–]
MRSA AH-1263			
—	—	—	**4**
NDM-335	>200	60	0.125 [32]
		30	0.125 [32]
		15	4 [–]
NDM-400	>200	60	0.015625 [256]
		30	0.125 [32]
		15	4 [–]
NDM-408	>200	60	2 [2]
NDM-427	>200	60	0.25 [16]
NDM-428	>200	60	2 [2]

Table 7 Cefoxitin MICs against MRSA ATCC BAA-1556, MRSA ATCC 43300, and MRSA AH-1263 in the presence of benzothiophene analogs

Compound	Standalone MIC (μM)	Concentration (μM)	Cefoxitin MIC (μg mL^{-1}) [fold reduction]
MRSA ATCC BAA-1556			
—	—	—	**32**
NDM-335	>200	60	16 [2]
NDM-400	>200	60	0.125 [256]
	>200	30	16 [2]
NDM-408	>200	60	16 [2]
NDM-427	>200	60	16 [2]
NDM-428	>200	60	32 [–]
MRSA ATCC 43300			
—	—	—	**32**
NDM-335	>200	60	32 [–]
NDM-400	>200	60	0.5 [64]
		30	16 [2]
NDM-408	>200	60	32 [–]
NDM-427	>200	60	16 [2]
NDM-428	>200	60	32 [–]
MRSA AH-1263			
—	—	—	**32**
NDM-335	>200	60	16 [2]
NDM-400	>200	60	16 [2]
NDM-408	>200	60	16 [2]
NDM-427	>200	60	8 [4]
NDM-428	>200	60	32 [–]

that each have one of the 15 nonessential *S. aureus* two component systems deleted, as well as a *mecA* transposon mutant, and activity was compared to that against the parent (MRSA AH-1263).[30] First, the oxacillin MIC was determined for each strain (Table 8). Four strains have significantly lower oxacillin MICs than the parent strain, indicating involvement of these gene products in oxacillin resistance. AH 2360 (Δ*graRS*),[30] AH 2087 (Δ*vraRS*),[30] AH 2090 (Δ*kdpDE*),[30] and AH 5929 (*mecA*:Tn mutant)[31] have oxacillin MICs of 0.5 μg mL^{-1} (64-fold), 4 μg mL^{-1} (eight-fold), 8 μg mL^{-1} (four-fold), and 0.25 μg mL^{-1} (128-fold), respectively. In all four strains, each compound has a standalone MIC >200 μM. Against the *graRS* mutant, no compounds potentiate the activity of oxacillin (MIC lowered no more than two-fold), suggesting that GraRS is required for adjuvant activity. Against the *vraRS* deletion strain AH 2087, all compounds further potentiate the activity of oxacillin, implying that the VraRS regulatory system is not the target of this series of compounds. Against the *kdpDE* deletion strain AH 2090, only **NDM-428** does not further potentiate oxacillin (MIC lowered only two-fold), indicating that KdpDE is not the general target for this series of compounds, but further investigation is required to determine if it plays a role in the activity of **NDM-428**. Finally, against the *mecA* transposon mutant AH 5929, no compound further potentiates the activity of oxacillin, suggesting that PBP2a is also required for adjuvant activity. In aggregate, this indicates that the potential targets for these compounds are involved in the GraRS two component signaling system and/or PBP2a (in both cases either direct binding or downregulation of the genes).

To follow up on these studies, we performed qRT-PCR to determine if the mechanism of the compounds was underpinned by downregulation of *mecA*, *graR*, or *graS* using **NDM-335** and BAA-1556 as our representative adjuvant/strain combination (Fig. 9). At all concentrations studied (6.25–50 μM), no statistically significant downregulation of any of the genes was observed, indicating that neither the regulatory genes driving transcription of *mecA*, nor the genes encoding the GraRS TCS are the targets of this compound.

To establish the therapeutic index (TI) of lead compounds, their effect on viability of the hepatocellular carcinoma cell line, HepG2, was determined. The TI for conventional antibiotic development is defined as (mammalian cell CC_{50})/(antibiotic MIC), with a TI ≥ 50 desirable for further development. Because adjuvants, by definition, are typically non-toxic to bacteria, we define adjuvant TI as (mammalian cell CC_{50})/MEC. The CC_{50} was determined for the five lead compounds (Table 9). The parent compound, **NDM-335**, has a CC_{50} of 96.8 μM, an active concentration of 7.5 μM, and a

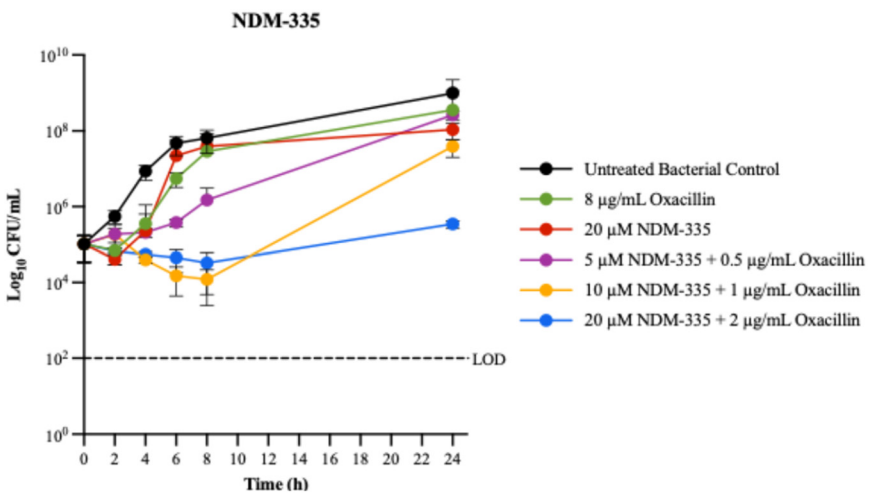


Fig. 6 MRSA 43300 growth curves for **NDM-335** and oxacillin. Data ± SD from at least three replicates.

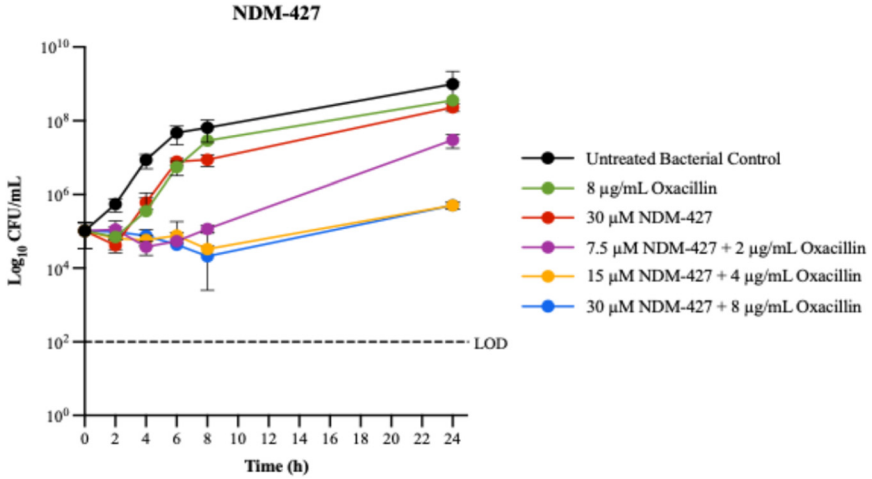


Fig. 7 MRSA 43300 growth curves for **NDM-427** and oxacillin. Data ± SD from at least three replicates.

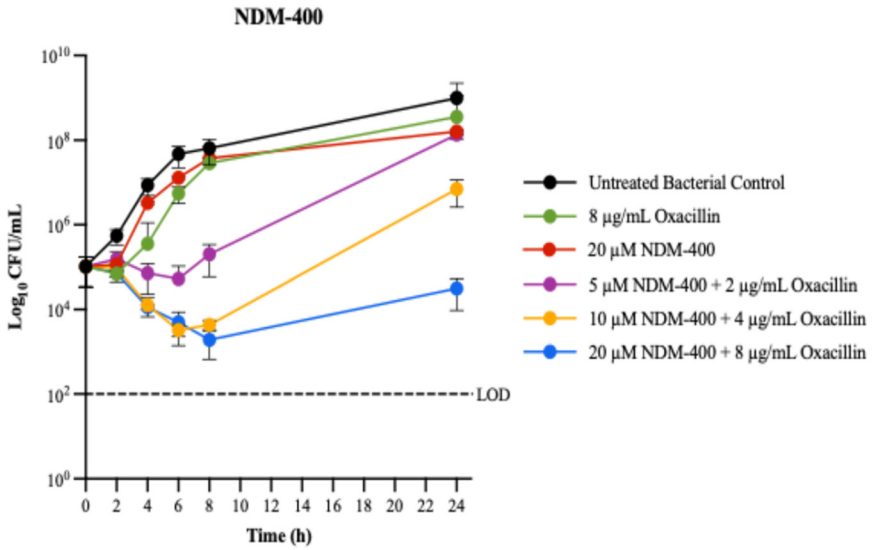


Fig. 8 MRSA 43300 growth curves for **NDM-400** and oxacillin. Data ± SD from at least three replicates.

TI of 12.9. **NDM-400** returns a CC_{50} of 145.9 μM, an active concentration ranging from 5 μM to 7.5 μM across the three strains, resulting in a TI ranging from 19.5 to 29.2. **NDM-408** has a CC_{50} of 81.4 μM, an active concentration of 5 μM, and a TI of 16.3. **NDM-427** returns a CC_{50} of 81.4 μM, an active concentration of 7.5 μM, and a TI of 10.9. Finally, **NDM-428** has a CC_{50} of 179.6 μM, an active concentration ranging from 5 μM to 7.5 μM, and a TI ranging from 24.0 to 35.9. While none of the compounds return a TI ≥ 50, all but one compound (**NDM-427**) has an increased TI compared to the parent compound.

Conclusion

Through an initial SAR study on benzothiophene **NDM-335**, we identified several novel compounds that lower the MIC of oxacillin to or below the CLSI breakpoint of 2 μg mL^{-1} against at least one MRSA strain at 10 μM or lower. Structural trends revealed a strong correlation between activity and sulfur-containing heterocycles, with benzothiophene and benzothiazole motifs providing the most robust adjuvant activity. Substitution patterns within the benzothiophene ring significantly influence potency and strain-based activity, underscoring the sensitivity of this scaffold to modifications. Activity was largely restricted to oxacillin and penicillin G, with limited potentiation of cefoxitin, suggesting antibiotic-class selectivity consistent with PBP resistance mechanisms. Preliminary mechanistic studies further support a role for PBP2a in the observed adjuvant activity. Loss of potentiation in a *mecA* transposon mutant and in a *graRS* deletion strain indicates that PBP2a and GraRS-mediated regulation are required for activity. Follow up qRT-PCR analysis has indicated neither controlling *mecA* expression, nor the genes encoding the GraRS TCS are the targets of these compounds.

Table 8 Lead compounds against a *mecA* transposon mutant and two TCS deletion strains

Compound	Standalone MIC (μM)	Concentration (μM)	Oxacillin MIC (μg mL^{-1}) [fold reduction]
AH 1263			
—	—	—	**32**
NDM-335	>200	7.5	0.25 [128]
NDM-400	>200	15	0.5 [64]
NDM-408	>200	60	8 [4]
NDM-427	>200	7.5	2 [16]
NDM-428	>200	7.5	0.5 [64]
AH 5929 (*mecA*:Tn)			
—	—	—	**0.25**
NDM-335	>200	7.5	0.125 [2]
NDM-400	>200	15	0.125 [2]
NDM-408	>200	60	0.125 [2]
NDM-427	>200	7.5	0.125 [2]
NDM-428	>200	7.5	0.25 [–]
AH 2087 Δ*vraRS*			
—	—	—	**4**
NDM-335	>200	7.5	0.5 [8]
NDM-400	>200	15	0.125 [32]
NDM-408	>200	60	1 [4]
NDM-427	>200	7.5	0.25 [16]
NDM-428	>200	7.5	1 [4]
AH 2090 Δ*kdpDE*			
—	—	—	**8**
NDM-335	>200	7.5	0.25 [32]
NDM-400	>200	15	0.25 [32]
NDM-408	>200	60	0.5 [16]
NDM-427	>200	7.5	0.25 [32]
NDM-428	>200	7.5	4 [2]
AH 2360 Δ*graRS*			
—	—	—	**0.5**
NDM-335	>200	7.5	0.5 [–]
NDM-400	>200	15	0.5 [–]
NDM-408	>200	60	0.5 [–]
NDM-427	>200	7.5	0.25 [2]
NDM-428	>200	7.5	0.5 [–]

We are currently purifying PBP2a from BAA-1556 to perform both binding and inhibition assays to further establish the mechanism of action of these compounds and will be reported in due course. This work validates the benzothiophene scaffold as a promising foundation for β-lactam adjuvant development and provides a framework for future efforts aimed at enhancing potency, maintaining appropriate safety metrics, and defining mechanistic activity. Compared to our previously identified PBP2a-dependent oxacillin adjuvants, the benzothiophenes appear more promising than the phenyl urea scaffold for further development as they remain active in potentiation assays at lower concentrations (10 μM or lower), while phenyl urea derivatives are active at high concentrations (60 μM).[18]

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article has been provided either within the manuscript or in the supplementary information (SI).

Supplementary information: the SI includes details on the bacterial strains, media and culture conditions used. Methods for determination of compound minimum inhibitory concentrations as well as antibiotic potentiation. General synthetic procedures are included as well as ^{1}H NMR data for known compounds. Full characterization is provided for all novel compounds which includes ^{1}H NMR/^{13}C NMR/HRMS/UV/IR data. A ^{1}H NMR spectra is provided for each novel compound. Compound crystallographic data has also been included. See DOI: https://doi.org/10.1039/d5md01109d.

CCDC 2514565 contains the supplementary crystallographic data for this paper.[32]

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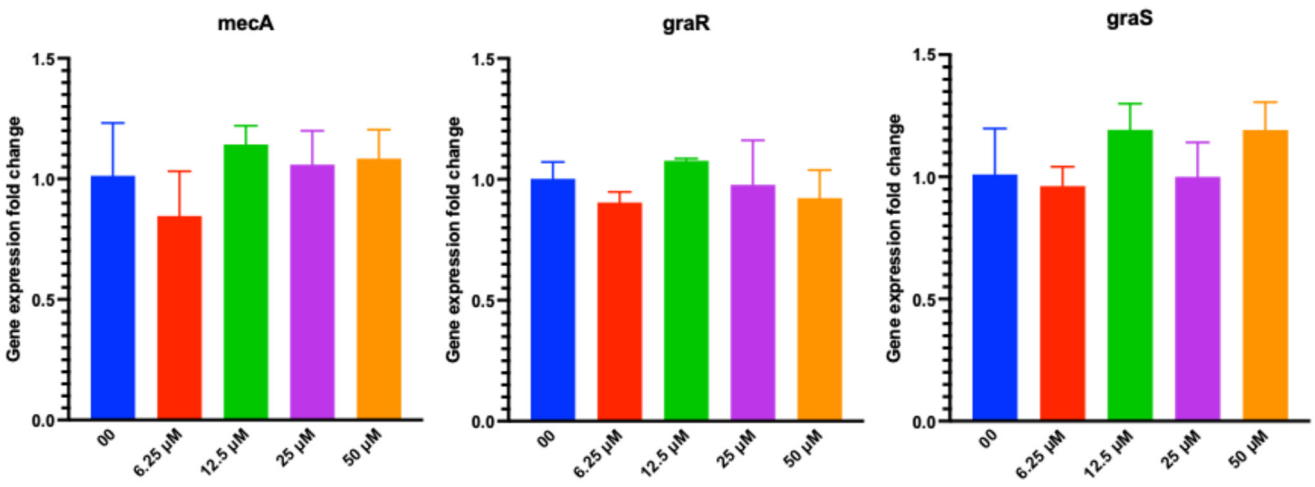

Fig. 9 qRT-PCR studies with varying concentrations of **NDM-335** against *mecA*, *graR*, and *graS*.

Table 9 HepG2 CC_{50}s (μM) and TIs

	NDM-335	NDM-400	NDM-408	NDM-427	NDM-428
CC_{50} (μM)	96.8 ± 12.0	145.9 ± 13.5	89.6 ± 1.9	81.4 ± 10.4	179.6 ± 29.98
TI	12.9	19.5–29.2	16.3	10.9	24.0–35.9

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