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The Discovery of 2-Aminobenzimidazoles That Sensitize *Mycobacterium smegmatis* and *M. tuberculosis* to β -Lactam Antibiotics in a Pattern Distinct from β -Lactamase Inhibitors

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Abstract

A library of 2-aminobenzimidazole derivatives was screened for the ability to suppress β -lactam resistance in *Mycobacterium smegmatis*. Several non-bactericidal compounds were identified that

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Conflict of interest

C.M. is a co-founder and member of the board of directors of Agile Sciences, a biotechnology company that is seeking to commercialize the antibiofilm and antibiotic sensitization activity of 2-aminoimidazoles and 2-aminobenzimidazoles.

reversed intrinsic resistance to β -lactam antibiotics in a manner distinct from β -lactamase inhibitors. Activity also translates to *M. tuberculosis*, with a lead compound from this study potently suppressing carbenicillin resistance in multiple *M. tuberculosis* strains (including multidrug-resistant strains). Preliminary mechanistic studies revealed that the lead compounds act through a mechanism distinct from that of traditional β -lactamase inhibitors.

Keywords

aminobenzimidazoles; antibiotic resistance; medicinal chemistry; tuberculosis

In 2014, *Mycobacterium tuberculosis* infected over nine million individuals and was responsible for 1.5 million deaths worldwide, making tuberculosis (TB) the leading cause of death from infections.^[1] Modern tuberculosis treatment consists of six to nine months of combination therapy with the first-line anti-TB drugs isoniazid, rifampicin, ethambutol, and pyrazinamide. Although this regimen is highly effective at treating drug-susceptible TB, it is ineffective at eradicating infections caused by multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis*.^[2] Treatment of MDR and XDR infections often requires prolonged use of second-line antibiotics that have significant side effects.^[3] In 2009, just a few years after the emergence of XDR *M. tuberculosis* strains, the World Health Organization described combating drug-resistant tuberculosis as one of the most “profound challenges facing global health”.^[4] There is a clear need for novel treatment strategies with improved patient tolerance that are effective against MDR and XDR strains.

The β -lactam class of antibiotics is one of the most important and widely prescribed classes of antimicrobial drugs. These antibiotics are generally considered as one of the safest classes of antibiotics available to treat Gram-positive and Gram-negative bacterial infections.^[5] Unfortunately, β -lactam antibiotics have historically been avoided in the treatment of tuberculosis because *M. tuberculosis* is intrinsically resistant to β -lactams owing to both a chromosomally encoded β -lactamase and a poorly permeable outer membrane.^[6] However, previous reports have demonstrated that the resistance of *M. tuberculosis* to β -lactam antibiotics can be reversed through adjunctive therapy with β -lactamase inhibitors.^[7]

Recently, we demonstrated that 2-aminoimidazole (2-AI) **1** was able to restore isoniazid susceptibility in *M. tuberculosis* expressing in vitro drug tolerance as a result of the formation of biofilms.^[8] One unexpected finding from this study was that compound **1** also sensitized planktonic cultures of *M. tuberculosis* to the effects of β -lactam antibiotics (Figure 1). We have previously demonstrated that certain members of our 2-aminoimidazole library have the ability to break acquired resistance (β -lactams and colistin) in MDR Gram-positive and Gram-negative bacteria.^[9] However, the observation of increased susceptibility of *M. tuberculosis* to the effects of β -lactam antibiotics was the first example of these small molecules breaking intrinsic resistance. Based upon our previous success in identifying antibiotic adjuvants, we posited that additional small molecules in our library could possess the ability to break intrinsic resistance to β -lactam antibiotics in mycobacteria.

Herein, we describe the identification of the N1-substituted 2-aminobenzimidazole (2-ABI) **2**^[10] as one such molecule (Figure 1). We report the results of structure–activity studies

based on using *M. smegmatis* as a surrogate strain. Furthermore, we demonstrate that this activity is distinct from that of β -lactamase inhibitors, and that the mechanism is not dependent upon direct inhibition of β -lactamase activity. Finally, we demonstrate that compound **2** is able to sensitize multiple *M. tuberculosis* strains to the effects of the representative β -lactam antibiotic carbenicillin.

We initiated this study by screening a subset of the compounds from our internal library for their ability to lower the minimum inhibitory concentration (MIC) of cefotaxime against our representative mycobacterium strain (*M. smegmatis* ATCC 700084, mc²155; see the Supporting Information for the full list of compounds screened). Only one compound from this screen, 2-ABI **2**, was identified as reducing the MIC of cefotaxime. This compound, which possesses a 3-phenylpropyl substituent at the N1 position of the 2-ABI, markedly reduced the cefotaxime MIC, namely from 128 to 1 $\mu\text{g mL}^{-1}$ (128-fold) at 20 μM (9 $\mu\text{g mL}^{-1}$).

The activity spectrum of compound **2** was then established by screening for activity in combination with a panel of β -lactam antibiotics (Table 1). Broad repotential activity was observed, with compound **2** reducing the MIC of all tested β -lactam antibiotics apart from cefadroxil. The degree of reduction ranged from fourfold to 256-fold, with the greatest effect observed for the cephalosporins cefaclor, cefotaxime, and ceftazidime (128- to 256-fold MIC reductions). Compound **2** effected more modest reductions in the MICs for other cephalosporins, including eightfold reductions in the MICs for cefoxitin and cephalothin and a 16-fold reduction in the MIC for cefmetazole. The MIC reductions for penicillin-type antibiotics ranged from fourfold to 32-fold.

To delineate the structure–activity relationship (SAR) of this class of compounds, we screened a library of previously reported analogues of compound **2**^[10] that possess a variety of N1 substituents (Figure 2) for the ability to reduce the MIC of cefotaxime against *M. smegmatis* (see the Supporting Information, Table S2). Compounds possessing aliphatic chains of differing lengths (compounds **3–8**) in place of the 3-phenylpropyl chain at the N1 position of compound **2** were not able to reduce the cefotaxime MIC against *M. smegmatis*. Compound **9**, which has a cyclopentane ring at the N1 position also did not effect any reduction in the cefotaxime MIC. Compounds with methoxy substituents at the *ortho*, *meta*, or *para* position of the aryl ring (compounds **10–13**) again were not able to lower the MIC of cefotaxime below 128 $\mu\text{g mL}^{-1}$. Compounds with linkers of various lengths at the N1 position (compounds **14–16**) also all failed to sensitize *M. smegmatis* to cefotaxime (0- to 2-fold reduction). Compound **17**, which has a 4-pentylbenzoyl substituent at the N1 position, similarly did not reduce the cefotaxime MIC. Finally, compounds in which the aryl ring was replaced with various other aromatic rings (compounds **18–22**) also all failed to reduce the MIC of cefotaxime, leading to the conclusion that the 3-phenylpropyl chain at the N1 position of **2** is crucial for potentiating the response of *M. smegmatis* to β -lactam antibiotics.

After exploring substituent effects at the N1 position, we then turned our attention to delineating SAR parameters at the tail of the molecule by synthesizing a series of

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compounds in which the N6 substituent was varied. The synthesis of N6-substituted 2-ABI derivatives was accomplished through the route outlined in Scheme 1 and the Supporting Information. Commercially available 4-fluoro-3-nitroaniline was protected with a Boc group, followed by nucleophilic aromatic substitution with 3-phenyl-1-propylamine to give **23**. This intermediate was subjected to transfer hydrogenation conditions, followed by condensation with cyanogen bromide and then Cbz protection to yield 2-ABI **24**. Following Boc deprotection, the introduction of N6 substituents was accomplished through acylation with the corresponding acid chloride or activated carboxylic acid to yield compounds generally depicted as **25**. The Cbz group was removed under hydrogenation conditions, and the target 2-ABIs **26–45** were isolated as their respective HCl salts (Figure 3). This approach yielded an array of 4-alkyl-substituted analogues (**26–31**) and compounds with alkoxybenzoyl (**32–34**), halogenated benzoyl (**35–38**), and aliphatic (**39–45**) substituents in the N6 position. Yields for all reactions can be found in the Supporting Information.

To directly compare the biological activities of the compounds in the N6 library to that of lead compound **2**, each compound was assayed at 20 μM in the presence of four β -lactam antibiotics: ampicillin, oxacillin, cefotaxime, and ceftiofur. Selected data are summarized in Table 2 and Table 3, and comprehensive data can be found in Table S2 and Table S3. Of the 4-alkyl-substituted analogues **26–31**, only compound **29**, which has a hexyl tail as opposed to the pentyl tail of compound **2**, was able to bring about any reduction in the MICs of the four antibiotics. Compound **29** reduced the MICs of cefotaxime and ceftiofur by factors of 64 and 2, respectively, as compared to the 128-fold and eightfold reductions effected by compound **2**. A twofold reduction in the MICs for ampicillin and oxacillin was observed for **29** compared to a 16-fold reduction in the presence of **2**. Shortening the aliphatic chain below the five-carbon chain of **2**, or lengthening it by more than one carbon atom, drastically reduced the ability of the compound to suppress antibiotic resistance. Of the alkoxybenzoyl compounds **32–34**, compound **33**, which possesses a butoxy tail, was the most potent, exhibiting similar activity to compound **2**. However, varying the alkoxy chain length from that of compound **33** also drastically reduced the biological activity, as shown with compounds **32** and **34**. The halogenated compounds **35–38** were unable to reduce the MICs of the four tested antibiotics. As these compounds lack a *para*-alkyl or -alkoxy chain on the aryl ring, the results suggest the importance of having either an alkyl or alkoxy chain of specific length attached to the N6 position of the 2-ABI ring. Of the aliphatic compounds **39–45**, only **41** demonstrated an ability to break resistance, with four- and eightfold reductions in the MICs for ampicillin and oxacillin, respectively, and 128-fold and fourfold reductions observed for cefotaxime and ceftiofur, respectively. In summary, this SAR study revealed that an aryl alkyl, aryl alkoxy, or alkyl group at the N6 position is required for potentiation, and that the chain length has a considerable effect on activity. Overall, the library delivered three analogues (along with lead compound **2**) that were able to reduce the MIC of four β -lactam antibiotics against *M. smegmatis*. Compound **33** was the only compound to exhibit similar activity to compound **2** for all four tested antibiotics.

We next compared our lead 2-ABI compounds to three FDA-approved β -lactamase inhibitors at the same concentration (20 μM) for their ability to reduce the MICs of the same four β -lactam antibiotics against *M. smegmatis* (Table 2 and Table 3). Sulbactam,

tazobactam, and potassium clavulanate brought about a 64- to 128-fold reduction for ampicillin compared to the 8- and 16-fold reduction brought about by compounds **33** and **2**, respectively. However, the β -lactamase inhibitors were not as effective at reducing the MICs of oxacillin, cefotaxime, and cefoxitin as compounds **2** and **33**. The β -lactamase inhibitors produced a twofold reduction for oxacillin and cefoxitin whereas compounds **2** and **33** produced a 16-fold reduction for both antibiotics. In the presence of the β -lactamase inhibitors, a four- to eightfold reduction in the cefotaxime MIC was observed in comparison to the 128-fold reduction brought about by compounds **2** and **33**. Overall, sulbactam, tazobactam, and clavulanate were more effective at lowering the MIC of the penicillin ampicillin in *M. smegmatis* while the 2-ABIs **2** and **33** produced a greater reduction in the MICs of oxacillin and the cephalosporins (cefotaxime and cefoxitin).

Prompted by this striking differential activity between the β -lactam classes, we decided to determine whether our 2-ABI compounds inhibited β -lactamase activity by conducting a nitrocefin hydrolysis assay (Figure S1).^[11] *M. smegmatis* was treated with compound **2** (20 μ M) for one hour; then, cells were collected and lysed by sonication. The cell lysates were dosed with 50 μ g mL⁻¹ of nitrocefin, and the absorbance at 486 nm was recorded every two minutes for four hours. Treatment of the cell lysates with 20 μ M of compound **2** alone or in combination with cefotaxime resulted in a hydrolysis rate that mirrored that of the untreated control. We also dosed untreated cultures post lysis with compound **2** and observed no difference in the rate of nitrocefin hydrolysis. For comparison, cell lysates were also dosed with sulbactam, tazobactam, and potassium clavulanate at 20 μ M, and a marked difference in absorbance was observed compared to the untreated control. The fact that the rate of nitrocefin hydrolysis is not affected by the presence of compound **2** suggests that compound **2** does not inhibit β -lactamase activity.

Time-kill curves were constructed to assess the effect of compounds **2** and **33** on cefotaxime activity over time (Figure S2). *M. smegmatis* was grown in the presence of either compound **2** or **33**, cefotaxime, or a combination of compound and cefotaxime. Samples were plated after 4, 8, 24, 28, 32, and 48 h. Compounds **2** and **33** were dosed at 20 μ M. At early time points (10–30 h), we observed very modest inhibition of bacterial growth when bacteria were dosed with **2** or **33** only; however, at > 36 h, the bacterial growth matched that of the control. At the 48 h time point, there is a < 0.1 log reduction in the number of colony-forming units (CFUs) when treated with 1 μ g mL⁻¹ of cefotaxime alone; however, when co-administered with 20 μ M of **2** or **33**, there is a 5.06 log reduction in the number of CFUs, equivalent to treatment with 512 μ g mL⁻¹ of cefotaxime alone. Comprehensive tables comparing the log reductions in CFUs can be found in the Supporting Information.

Next, we studied the ability of compound **2** to suppress β -lactam resistance in *M. tuberculosis*. *M. smegmatis* is typically employed as a surrogate for *M. tuberculosis* owing to the difficulty associated with handling *M. tuberculosis* strains compared to *M. smegmatis* (BSL3 vs. BSL1); however, there have been questions about the value of this approach.^[12] Although we do not debate this view, the activities of our compounds typically translated from *M. smegmatis* to *M. tuberculosis*. Given this new activity, we wanted to again establish that *M. smegmatis* was indeed a valid screening surrogate to be employed as a proxy to identify compounds from our library that would be active against a variety of *M.*

tuberculosis strains. To this end, we studied the ability of compound **2** to lower the carbenicillin MIC against four *M. tuberculosis* strains: H37Rv, CSU 18, CSU 32, and CSU 81 (Table 4). H37Rv is a standard, drug-susceptible *Mtb* laboratory strain that is routinely employed for screening and genetic work. The CSU strains are drug-resistant clinical isolates; CSU 18 is isoniazid-resistant, CSU 81 is rifampin-resistant, and CSU 32 is an MDR strain (isoniazid/rifampin-resistant). The carbenicillin MIC is 256 $\mu\text{g mL}^{-1}$ against each strain. However, in the presence of compound **2** (32 μM), the carbenicillin MICs were significantly reduced, returning values of 8, 2, 0.6, and 25 $\mu\text{g mL}^{-1}$ for strains H37RV, CSU 18, CSU 32, and CSU 81, respectively, which corresponds to a 10- to 427-fold increase in antibiotic activity. Most notably, the greatest reduction in MIC was observed for the MDR strain, CSU 32.

Finally, to investigate the suitability of 2-ABIs for in vivo studies, we explored the safety profiles of the four lead compounds. Compounds **2**, **29**, and **33** effected less than 10 % hemolysis at concentrations up to 500 μM while compound **41** effected only 13 % (Table S6). We also evaluated the whole organism toxicity in *Galleria melonella*, and at concentrations up to 400 mg kg^{-1} , we observed 97% survival (Figures S4–S7).

In conclusion, after screening and evaluation of an initial library of 22 N1-substituted 2-ABIs for suppression of β -lactam resistance in *M. smegmatis*, compound **2** was identified as a lead compound for further evaluation. This 2-ABI lowered the cefotaxime MIC by a factor of 128, and also reduced the MICs of many other clinically used β -lactam antibiotics by factors of 8 to 256. Based on compound **2**, 19 additional N6-substituted 2-ABIs were synthesized and evaluated for their ability to suppress antibiotic resistance. From this second-generation library, three compounds reduced the MICs of the four β -lactam antibiotics tested. Compound **33** was able to effect similar reductions as compound **2**. Time-kill curves showed that the lead compounds had limited bacterial toxicity alone at the tested concentration (20 μM). Compound **2** displayed potent synergistic activity with cefotaxime, with a > 5 log reduction in CFU mL^{-1} observed after 48 h. Interestingly, we found that these compounds have a β -lactam potentiation spectrum that is orthogonal to that of traditional β -lactamase inhibitors. This was further verified by establishing that active 2-ABIs had no effect on the β -lactamase activity in *M. smegmatis*, indicating that these compounds act through a mechanism distinct from that of traditional β -lactamase inhibitors. Finally, we established that the activity translates to the important pathogen *M. tuberculosis*, for which new treatment options are vitally needed, with the lead compound sensitizing several multidrug-resistant *M. tuberculosis* strains to the effects of carbenicillin by factors of > 427 . Investigations into the exact mechanism of action of the β -lactam sensitization of our 2-ABI compounds are currently ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.
2-Aminoimidazole **1** and 2-aminobenzimidazole **2**.

Figure 2.
N1-substituted 2-ABIs.

Figure 3.
N6-substituted 2-ABIs.

Scheme 1.

Synthesis of N6-substituted 2-ABIs. Reagents and conditions: a) Boc_2O , Et_3N , DMAP, THF, RT, 16 h; b) 3-phenyl-1-propylamine, EtOH, reflux, 24 h; c) NH_4HCO_2 , Pd/C, EtOH, reflux, 2 h; d) BrCN , CH_2Cl_2 , RT, 24 h; e) CbzCl , $\text{Sc}(\text{OTf})_3$, Et_3N , CH_2Cl_2 , 0°C to RT; f) TFA/ CH_2Cl_2 (1:4), 0°C to RT; g) RCOCl , Et_3N , CH_2Cl_2 , RT, 1 h or RCOOH , EDC, HOBT, Et_3N , CH_2Cl_2 , RT, 16 h; h) NH_4HCO_2 , Pd/C, EtOH, reflux, 2 h; i) 3 M HCl, MeOH. Boc = *tert*-butoxycarbonyl, Cbz = benzyloxycarbonyl, DMAP = 4-dimethylaminopyridine, EDC = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, HOBT = 1-hydroxybenzotriazole, Tf = trifluoromethanesulfonyl, TFA = trifluoroacetic acid.

Table 1:

Suppression of β -lactam antibiotic resistance in *M. smegmatis* (ATCC 700084, mc²155) by compound **2**.

Antibiotic	MIC ^[a] without 2	MIC ^[a] with 2 [20nM]	Fold reduction
ampicillin	256	16	16
carbenicillin	1024	128	8
methicillin	512	32	16
naficillin	512	16	32
oxacillin	256	16	16
penicillin G	512	16	32
piperacillin	512	128	4
cefaclor	256	1	256
cefadroxil	1024	1024	0
cefmetazole	8	0.5	16
cefotaxime	128	1	128
cefoxitin	16	2	8
ceftazidime	512	2	256
cephalothin	1024	128	8

^[a] All MIC values are given in $\mu\text{g mL}^{-1}$.

Table 2:

Select antibiotic repotential data for N6-substituted 2-ABIs and β -lactamase inhibitors against *M. smegmatis*.

Compound ^[a]	Ampicillin MIC ^[b]	Fold reduction	Oxacillin MIC ^[b]	Fold reduction
–	256	–	256	–
2	16	16	16	16
29	128	2	128	2
33	32	8	16	16
41	64	4	32	8
sulbactam	4	64	128	2
tazobactam	2	128	128	2
potassium	2	128	128	2
clavulanate				

^[a] All compounds were tested at 20 μ M.

^[b] MIC values given in μ g mL^{–1}.

Table 3:

Select antibiotic repotential data for N6-substituted 2-ABIs and β -lactamase inhibitors against *M. smegmatis*.

Compound ^[a]	Cefotaxime MIC ^[b]	Fold reduction	Cefoxitin MIC ^[c]	Fold reduction
–	128	–	16	–
2	1	128	2	8
29	2	64	8	2
33	1	128	2	8
41	1	128	4	4
subactam	32	4	8	2
tazobactam	16	8	8	2
potassium	32	4	8	2
clavulanate				

^[a] All compounds were tested at 20 μ M.

^[b] MIC values reported in μ g mL^{–1}.

Table 4:

Suppression of carbenicillin resistance in *M. tuberculosis* strains by compound **2**.

Strain	Carbenicillin MIC ^[a] without 2	Carbenicillin MIC ^[a] with 2 [32 µM]	Fold reduction
H37Rv	256	8	32
CSU18	256	2	128
CSU32	256	0.6	427
CSU81	256	25	10

^[a] All MIC values are given in µg mL⁻¹.