A small molecule interacts with pMAC-derived hydroperoxide reductase and enhances the activity of aminoglycosides

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Abstract
The threat of antimicrobial resistance calls for more efforts in basic science, drug discovery, and clinical development, particularly gram-negative carbapenem-resistant pathogens. We sought to identify novel antibacterial agents against Acinetobacter baumannii ATCC19606 using whole cell-based screening. A small molecule named 6D1 with the chemical structure of 6-fluorobenzo[d]isothiazol-3(2H)-one was identified and exhibited activity against A. baumannii ATCC19606 strain (minimal inhibitory concentration, MIC = 1 mg l⁻¹). The mutation in the plasmid-derived ohrB gene that encodes a peroxidase was identified in spontaneously resistant mutants. Treatment of the bacteria with 6D1 resulted in increased sensitivity to peroxide, such as tert-butyl hydroperoxide. The binding of 6D1 and OhrB was confirmed by surface plasmon resonance. Interestingly, the MIC of kanamycin and gentamicin against spontaneously resistant mutants decreased. Finally, we identified the effect of 6D1 on enhancing the antibacterial activity of kanamycin and gentamicin, including against New Delhi metallo-β-lactamase (NDM-1)-producing carbapenem-resistant Klebsiella pneumoniae, but not in strains carrying aminoglycosides resistance genes. In this study, we identified a small molecule that suppresses the growth of A. baumannii, interacts with hydroperoxide reductase from A. baumannii ATCC19606 plasmid pMAC, and enhances the antibacterial activity of kanamycin and gentamicin. We propose that peroxidase may be potentially used as a target for aminoglycosides adjuvant development.

Introduction
Gram-negative pathogens, such as Acinetobacter baumannii, K. pneumoniae, and Pseudomonas aeruginosa have become resistant to almost all commonly used antimicrobial agents, including aminoglycosides, quinolones, and broad-spectrum β-lactams. Overall, for instance, ~45% of all global A. baumannii isolates are considered multidrug-resistant (bacteria resistant to more than three antibiotic classes) [1, 2].

With the emergence of carbapenem-resistant gram-negative pathogens, such as A. baumannii (CRAB), tigecycline and polymyxin-class antibiotics are the only currently available treatment options [3]. However, treatment outcomes of tigecycline have been hampered by the low serum concentrations of the drug in the approved dosing regimen and the low penetration in the epithelial lining fluid of mechanically ventilated patients [4]. The resistance of polymyxin-class antibiotics as well as nephrotoxicity and neurotoxicity are the major factors that limit the usage of polymyxin. Previous clinical observations showed that the rates of nephrotoxicity occurred in ~60% of patients who received colistin or polymyxin B therapy [5–7]. Eravacycline, cefiderocol, and
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plazomicin seem to be promising new agents against A. bauman
ni. However, evaluation of their position in clinical prac
tice and particularly in ventilator-associated pneumonia has not
been performed to date [8, 9].

The present clinical pipeline does not meet current needs,
and thus more investment is required in basic science, drug
discovery, and clinical development, particularly gram-
negative carbapenem-resistant pathogens, including CRAB
[10]. Therefore, we launched a whole cell-based screening
program for A. baumannii. Here, we report the discovery of
compound 6D1 that exhibits anti-A. baumannii activity. In
addition, we show the enhancement effect of 6D1 on the
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Materials and methods

Bacterial strains, growth conditions, reagents, and
screening strategy

A. baumannii, K. pneumoniae, and P. aeruginosa were grown
in liquid broth (LB) medium or LB agar. Antibiotic (pur-
chased from Sigma-Aldrich, USA) solutions were prepared at
a concentration of 1 mg ml
−1 in distilled water or 100%
dimethylsulfoxide (DMSO), filter-sterilized, and frozen at
−20 °C until use. The 100,000 compounds from Topscience
Co. to be screened were dissolved in 100% DMSO and stored
as frozen stocks at a concentration of 1 mg ml
−1.

We sought anti-A. baumannii compounds by testing com-
 pounds for inhibition of A. baumannii ATCC19606. A whole-
cell assay was used because of its ability to concurrently assess
multiple targets. Compounds were prepared in 96-well plates
at a concentration of 10 mg l
−1 in 50 μl LB broth. A 50-μl
aliquot of each bacteria culture was then added to each well of
the 96-well plate at an OD
600 = 0.006. The plates were incu-
bated overnight at 37 °C, and the primary active hits were
filtered by achieving at least 90% of bacterial growth inhibi-
tion using Cell Counting Kit-8 (MCE, USA). Subsequently,
twofold serial dilutions of primary hits were prepared for the
determination of minimal inhibitory concentration (MIC),
deﬁned as the lowest concentration of compound that inhibited
90% of bacterial growth. Compounds with an MIC ≤ 1 mg l
−1
were selected for further investigation.

Spontaneously resistant mutant selection

Spontaneously resistant mutants were selected via stepwise
exposure to increasing concentrations of the compounds.
An aliquot of mid-log phase (OD
600 = 0.6) bacterial culture
(1 ml) was added to 2 ml of medium containing serial
increasing concentrations of 6D1 until no growth was
observed. The bacteria that survived in culture were spread
onto agar plates containing the corresponding concentra-
tions of the 6D1 compound. All colonies that originated
from different plates and represent independent biological
events were subjected to whole-genome sequencing (WGS).
The resistance phenotype to the compound was conﬁrmed
by testing for a shift in MIC values.

WGS

Genomic DNA was extracted from each isolate using a
gram-negative bacterial genome extraction kit (Tiangen,
China). Whole-genome fragment libraries were prepared
using a paired-end sample preparation kit (Illumina, USA).
The genomes were sequenced using Illumina HiSeq 2500
platform (Illumina, USA) and assembled with de novo
SPAdes Genome Assembler (version 3.12.0) [11]. The
resulting reads were mapped to the A. baumannii
ATCC19606 reference genome, and mutations were iden-
tiﬁed using Snippy (https://github.com/tseemann/snippy).

The Contig ﬁles supporting the ﬁndings of the present
study have been deposited in the National Center for Bio-
technology Information’s Sequence Read Archive with
accession number PRJNA649401.

Effect of compounds on the tolerance of bacteria to
toxides

The effects on the tolerance of bacteria to peroxides were
determined by testing for shifts in MIC of tert-buty1
hydroperoxide (t-BHP), cumene hydroperoxide (CHP), and
hydrogen peroxide (H2O2) in the presence of the compound.

Protein expression and puriﬁcation

The cDNA encoding for full-length OhrB (DJ41_RS22870)
was chemically synthesized with codon optimization for
expression in E. coli. Vector pET-28a(+) was used for protein
expression. The construct was introduced into E. coli BL21
(DE3) cells for expression of recombinant proteins. The
recombinant strains were cultured in LB medium (10 g l
−1
NaCl, 10 g l
−1 tryptone, and 5 g l
−1 yeast extract) at 37 °C,
220 rpm until the OD600 reached 0.6. The cell cultures were
then supplemented with 0.5 mM isopropyl β-D-1-thiogal-
lactopyranoside. The induced cells were further grown at
16 °C with shaking at 220 rpm overnight to induce the
expression of the recombinant protein.

To purify the recombinant proteins, the cells were har-
vested by centrifugation at 2000 × g for 30 min at 4 °C. The
pellet was resuspended in the lysis buffer containing 50 mM
NaHPO4 (pH 8.0), 0.3 M NaCl, 1 mM PMSF, 75 mM
MgCl2, and DNAase I (20 μg ml
−1) lysed by ultrasonication.
The lysate was then centrifuged at 23,000 × g for 30 min
at 4 °C, and the supernatant was loaded onto a Ni-NTA column.
(Qiagen, Germany). The target protein was eluted in buffer containing 0.3 M NaCl, 50 mM NaHPO4 (pH 8.0), and 250 mM imidazole. After sample analysis with SDS-PAGE, the fractions containing pure protein were pooled and concentrated to 0.5 ml, and then loaded onto a Superdex 200 increase column (GE Healthcare, USA). The protein concentration was determined using the Bradford method.

**Surface plasmon resonance (SPR) experiment**

OhrB were covalently immobilized to a sensor chip CM5 (29-1049-88, Sweden) by means of amino coupling. The running buffer used in the experiment contains 20 mM HEPES pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.1% DMSO, and the 6D1 compound was also dissolved in the running buffer. The sensor chip was washed with running buffer between each concentration. Reference runs were performed with blank (sensor chip only) and active (sensor chip with OhrB only) channel on the same sensor chip. The assay curves were constructed using serial concentrations of 6D1 of 7.5, 15, 30, 60, and 120 μM.

The kinetic parameters of the interaction and the affinity constants were calculated using Biacore T200 evaluation software.

**Checkerboard titration assay**

Drug interactions between 6D1 and the bactericidal drugs were performed using a chequerboard titration assay [12]. The fractional inhibitory concentration (FIC) was calculated using the following formula: (MIC of drug A or B in combination)/(MIC of drug A or B alone). The FIC index (FICI) was determined by adding the two FICs. Synergy, antagonism, and no interaction were defined as FICI ≤ 0.5, FICI > 4.0, and FICI = 0.5–4.0, respectively [12].

**Results**

**In vitro activity of 6D1**

We identified an active hit named 6D1 (MIC = 1 mg l⁻¹) with the structure of 6-fluorobenz[d]isothiazol-3(2H)-one, which is similar to 1,2-benzisothiazolin-3-one (BIT) and an antifungal drug ticlatone (Fig. 1). A moderate antibacterial activity of 6D1 was also observed against S. aureus (MIC = 2.5 mg l⁻¹), but not against K. pneumoniae, and P. aeruginosa (MIC ≥ 5 mg l⁻¹). Unexpectedly, MICs of 6D1 were high in CRAB clinical isolates (MIC = 5–10 mg l⁻¹) (Table 1).

**OhrB mutations confer resistance to 6D1**

To identify the target of 6D1, we obtained two 6D1 spontaneously resistant strains (3M and 5M) from independent cultures with bacterial growth in LB broth containing 10× MIC (10 mg l⁻¹) of 6D1. An increase in MIC indicated the resistance phenotype of 3M and 5M to 6D1 (Table 1). Compared with the wild-type (WT) parent strain A. baumannii ATCC19606, mutations located in plasmid (pMAC)-derived ohrB were identified both in 3M and 5M strains, resulting in the conversion of arginine at the position 15 (Arg15) to His or Cys of OhrB (Table 2). Molecular dynamics simulations and in silico mutagenesis

![Fig. 1 Chemical structure of 6D1 and its analogs. a 6D1, b 1,2-benzisothiazolin-3-one (BIT), c Ticlatone](image-url)
indicated that the corresponding Arg19 in Ohr from *Xylella fastidiosa* contributed to the stabilization of XfOhr in the closed state, suggesting that the mutations in 3M and 5M probably affect the function of OhrB [13].

6D1 reduces the tolerance of bacteria to peroxides

Ohr was first described in *Xanthomonas campestris*. It has since been found in a number of bacterial species [14, 15]. Owing to the Cys-based, thiol-dependent peroxidase activity, Ohr plays a central role in bacterial responses against fatty acid hydroperoxides and peroxynitrite, thus resulting in an “organic hydroperoxide resistance” phenotype [16]. Table 3 shows that the MICs of *t*-BHP, CHP, and H2O2 against *A. baumannii ATCC19606* were at least eightfold lower when in the presence of 2.5 mg l\(^{-1}\) of 6D1. The magnitude of MIC reduction coincided with the substrate preference of Ohr (H2O2 «« CHP < *t*-BHP) as previously reported [16]. In contrast, against 3M strain, the MICs of *t*-BHP, CHP, and H2O2 were almost not affected by the presence of 6D1; whereas against 5M strain, the MICs of *t*-BHP, CHP, and H2O2 were reduced, indicating that 6D1 could still affect the function of OhrB in 5M strain. However, the MICs of *t*-BHP and H2O2 against 3M or 5M strains were slightly lower than the WT, suggesting that the ohrB mutation resulted in reduced tolerance to peroxides. Moreover, growth retardation was also observed in 3M and 5M strains, but the growth of 3M strain was not affected by 6D1 (Fig. 2).

**MICs of bactericidal drugs in 3M and 5M strains**

With aim to identify the effects of OhrB protein function on the activity of bactericidal agents, we tested the MICs of a handful of bactericidal drugs against the 3M, 5M, and WT strains. Compared with the WT, the MICs of kanamycin and gentamicin against 3M and 5M strains decreased by at least twofold, thereby suggesting the association between OhrB function and aminoglycosides activity (Table 4).

**Drug combinations study**

The observed changes of MIC for kanamycin and gentamicin in 3M and 5M suggest that 6D1 probably enhances antibacterial activity. Therefore, we used a checkerboard titration assay to identify the drug interaction of 6D1 with kanamycin and gentamicin in different species. Table 5 revealed a synergistic effect between 6D1 and aminoglycosides against *K. pneumoniae* and *A. baumannii* (FICI = 0.5). Clinical isolates of carbapenem-resistant *K. pneumoniae* (CRKP) were also selected to test the activity of 6D1 and aminoglycosides combination. The results showed that 6D1 did not reverse the antibacterial activity of kanamycin and gentamicin in CRKP containing the aminoglycosides resistance gene. However, we found that 6D1 enhanced the activity of kanamycin and gentamicin against a strain of CRKP that harbored the NDM-1 gene (Tables 5 and S1).

**The interaction between 6D1 and OhrB**

To characterize the binding of 6D1 and OhrB, we first obtained the purified His-tag-fused recombinant OhrB protein. The interaction between 6D1 and OhrB was confirmed by SPR analysis. It demonstrated the binding of 6D1 to OhrB, with an
association rate constant of $k_a$ $2.33 \times 10^3 \text{M}^{-1}\text{s}^{-1}$, a dissociation rate constant $k_d$ $2.28 \times 10^{-3} \text{s}^{-1}$, and an equilibrium dissociation constant $K_D$ $9.79 \times 10^{-6} \text{M}$ (Fig. 3).

**Discussion**

Bacteria have evolved complex mechanisms to detoxify reactive oxygen species and thus strictly control H$_2$O$_2$ levels. A 9540-bp plasmid pMAC carried by *A. baumannii* ATCC19606 that contains an OhrB coding region conferred bacterial resistance to organic peroxide-generating compounds CHP and t-BHP was reported in 2006 [17]. In this study, a whole-cell assay revealed a compound 6D1, which imparted inhibitory effects on *A. baumannii* ATCC19606 and had a similar structure to BIT and the antifungal tictalone. The mutation site of the spontaneously resistant mutant and SPR result suggested that the interaction between 6D1 and pMAC-derived OhrB. A previous study suggested that cellular thiol groups are major targets of BIT [18]. The antimicrobial activity of 6D1 on *K. pneumoniae* was also reported previously [19]. Therefore, it provides a rationale that 6D1 acts on the thiol groups, thereby exerting an antibacterial effect. However, our study found that 6D1 can interact with Ohr. This is concordant with our result that 6D1 sensitizes *A. baumannii* ATCC19606 to CHP and t-BHP. Although Ohr is not an essential gene, and therefore cannot be the primary target of an antibiotic, the inhibitory effect of Ohr still suggests the potential application of 6D1, given its important role in bacterial resistance to peroxides.

In addition to developing antibiotics with new chemical structures and acting mechanisms, antibiotic adjuvants offer an alternative approach to combat resistance [20]. In this study, 6D1 was found to impart an inhibitory effect on OhrB, and thus it is reasonable to use this as an adjuvant in combination with other antibiotics that induce bacteria to produce hydroperoxides. In addition, a previous study showed that all bactericidal antibiotics induce protective responses to reactive oxygen species [21]. This suggests the potential of 6D1 as an adjuvant for bactericidal drugs. However, our data showed that 6D1 only enhances the activity of the aminoglycosides,
kanamycin, and gentamicin, but not non-aminoglycoside. This may be related to the reactivity order of Ohr to different peroxides, in which it mainly modulates the levels of fatty acid hydroperoxides and peroxynitrite [16]. Because the synergistic effect of 6D1 is achieved by inhibiting OhrB, it is not surprising that 6D1 was not effective on drug-resistant strains that harbored aminoglycosides resistance genes, such as the 16s rRNA methylase enzyme rmtB. Notably, the combination of 6D1 with kanamycin and gentamicin were effective on the CRKP strain carrying NDM-1. A recent study has shown that in Northeast China, the aminoglycoside resistance gene rmtB was detected in 96.61% of KPC-2-producing CRKP and in 21.74% of NDM-1-producing CRKP, indicating the potential combinative application of aminoglycosides and a peroxidase inhibitor, such as 6D1 in about 80% of NDM-1-producing CRKP [22].

The clinical use of aminoglycosides has been limited by its well-known toxicity and side effects, such as ototoxicity. Our study revealed the feasibility of enhancing the activity of aminoglycosides by inhibiting the detoxification ability of bacteria to peroxides, thereby providing a new target and strategy for the development of aminoglycoside enhancers in the near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. US Department of Health and Human Services: Centers for Disease Control and Prevention. Antibiotic Resistant Threats In The United States. Centers for Disease Control and Prevention; 2019. https://www.cdc.gov/drugresistance/biggest-threats.html.
2. Magil SS, et al. Multistate point-prevalence survey of health care-associated infections. N. Engl J Med. 2014;370:1198–208.
3. Garnacho-Montero J, et al. Task force on management and prevention of Acinetobacter baumannii infections in the ICU. Intensive Care Med. 2015;41:2057–75.
4. Giamarelou H, Poulakou G. Pharmacokinetic and pharmacodynamic evaluation of tigecycline. Expert Opin Drug Metab Toxicol. 2011;7:1459–70.
5. Xie R, Zhang XD, Zhao Q, Peng B, Zheng J. Analysis of global prevalence of antibiotic resistance in Acinetobacter baumannii infections disclosed a faster increase in OECD countries. Emerg Microbes Infect. 2018;7:31.
6. Qureshi ZA, et al. Colistin-resistant Acinetobacter baumannii: beyond carbapenem resistance. Clin Infect Dis. 2015;60:1295–303.
7. Hartzell JD, et al. Nephrotoxicity associated with intravenous colistin (colistimethate sodium) treatment at a tertiary care medical center. Clin Infect Dis. 2009;48:1724–8.
8. Connors KP, et al. Phase I, open-label, safety and pharmacokinetic study to assess bronchopulmonary disposition of intravenous eravacycline in healthy men and women. Antimicrob Agents Chemother. 2014;58:2113–8.
9. Poulakou G, Lagou S, Karageorgopoulos DE, Dimopoulos G. New treatments of multidrug-resistant Gram-negative ventilator-associated pneumonia. Ann Transl Med. 2018;6:423.
10. World Health Organization. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. World Health Organization; 2017. https://apps.who.int/iris/handle/10665/258965.
11. Bankevich A, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77.
12. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 2003;52:1.
13. Piccirillo E, et al. Structural insights on the efficient catalysis of hydroperoxide reduction by Ohr: Crystallographic and molecular dynamics approaches. PLoS One. 2018;13:e0196918.
14. Mongkolsuk S, Prairatana W, Loprasert S, Fuangthong M, Chamnongpol S. Identification and characterization of a new organic hydroperoxide resistance (ohr) gene with a novel pattern of oxidative stress regulation from Xanthomonas campesiris pv. phascoli. J Bacteriol. 1998;180:2636–43.
15. Atichartpongkul S, et al. Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. Microbiology. 2001;147:1775–82.
16. Alegria TG, et al. Ohr plays a central role in bacterial responses against fatty acid hydroperoxides and peroxynitrite. Proc Natl Acad Sci USA. 2017;114:E132–41.
17. Dorsey CW, Tomaras AP, Actis LA. Sequence and organization of pMAC, an Acinetobacter baumannii plasmid harboring genes involved in organic peroxide resistance. Plasmid. 2006;56:112–23.
18. Cöllier PJ, et al. Chemical reactivity of some isothiazolone biocides. J Appl Bacteriol. 1990;69:578–84.
19. Carmellino ML, Pagani G, Pregnolato M, Terreni M, Pastoni F. Antimicrobial activity of fluorinated 1,2-benzisothiazol-3(2H)-ones and 2,2′-dithiobis(benzamides). Eur J Med Chem. 1994;29:743–51.
20. Wright GD. Antibiotic adjuvants: rescuing antibiotics from resistance. Trends Microbiol. 2016;24:862–71.
21. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. Cell. 2007;130:797–810.
22. Lin L, Xiao X, Wang X, Xia M, Liu S. In vitro antimicrobial susceptibility differences between carbapenem-resistant K PC-2-producing and NDM-1-producing Klebsiella pneumoniae in a Teaching hospital in Northeast China. Micro Drug Resist. 2020;26:94–99.