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Identification and characterization of novel isothiazolones with potent bactericidal activity against multi-drug resistant *Acinetobacter baumannii* clinical isolates

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ABSTRACT

Acinetobacter baumannii has emerged as a globally important nosocomial pathogen characterized by an increased multi-drug resistance (MDR), leaving limited options for treating its infection. To identify novel antibacterial compounds with activity against clinical isolates of *A. baumannii*, we performed high-throughput screening against a chemical library of 42,944 compounds using nonpathogenic *Escherichia coli* MG1655 and identified 55 hit compounds. The antibacterial activities of 30 pure compounds were determined against MDR clinical isolates of *A. baumannii* obtained from Los Angeles County hospitals. Two isothiazolones identified, 5-chloro-2-(4-chloro-3-methylphenyl)-4-methyl-3(2H)-isothiazolone (Compound **6**) and 5-chloro-2-(4-chlorophenyl)-4-methyl-3(2H)-isothiazolone (Compound **7**), possess novel structure and exhibited consistent, potent and cidal activity against all 46 MDR *A. baumannii* clinical isolates and reference strains. Additionally, structure–activity relationship analysis involving several additional isothiazolones supports the link between a chloro-group on the heterocyclic ring or a fused benzene ring and the cidal activity. Attempts to obtain isothiazolone resistant mutants failed, consistent with the rapid cidal action and indicative of a complex mechanism of action. While cytotoxicity was observed with Compound **7**, it had a therapeutic index value of 28 suggesting future therapeutic potential. Our results indicate that high-throughput screening of compound libraries followed by in vitro biological evaluations is a viable approach for the discovery of novel antibacterial agents to contribute in the fight against MDR bacterial pathogens.

1. Introduction

Acinetobacter baumannii, a major cause of hospital and community-acquired infections worldwide, has been recently named a critical 'priority pathogen' by the World Health Organization due to the threat that it poses to human health [1]. There are *A. baumannii* strains that are resistant to nearly all major classes of antibiotics normally used to treat infections caused by this bacterium, including β-lactams, aminoglycosides, fluoroquinolones, chloramphenicol, tetracyclines, and rifampin [2–4]. In particular, increased incidences of multi-drug resistant (MDR) *A. baumannii* infections have been reported in military medical facilities treating U.S. military personnel injured in Iraq and Afghanistan, and civilian patients [5,6]. The omnipresence of these MDR *A. baumannii* strains leaves limited clinical options for treating infections caused by this pathogen, underscoring the need to develop novel antibiotics for bacterial pathogens in general and for *A. baumannii* in particular [7,8]. The exhaustion of natural antibiotics along with the rise of resistance has hindered our ability to deliver promising new drug candidates [9]. The advancement of laboratory robotics has led to the application of high-throughput screening (HTS) approaches for the discovery of inhibitors of bacterial growth [10-12].

In this communication, we report the identification of antibacterial inhibitors via HTS using Gram-negative *Escherichia coli* MG1655 as a screening target organism. Thirty compounds thus identified were tested against a panel of MDR clinical isolates of *A. baumannii*. Structure–activity relationship (SAR) analysis,

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cidality, resistance emergence and cytotoxicity of a series of potent isothiazolone inhibitors were investigated with the acquisition of several additional analogs. The results of this study provide additional insight into the effectiveness of isothiazolone compounds in targeting Gram-negative pathogens such as MDR *A. baumannii*. Thus, the approach of combining HTS and detailed biological evaluations of discovered hit compounds will facilitate the discovery of new therapeutic agents for the treatment of MDR bacterial pathogens.

2. Materials and methods

2.1. Bacterial strains and isolates

E. coli K-12 MG1655 (ATCC) was used as the target screening strain for HTS to obtain hit compounds with activities against Gram-negative bacteria. Quality control (QC) for antimicrobial susceptibility testing was carried out with the reference strains *E. coli* ATCC#25922, *Pseudomonas aeruginosa* ATCC#27853, *Enterococcus faecalis* ATCC#29212 and *Staphylococcus aureus* ATCC#29213 (Table 1). *A. baumannii* ATCC#19606 was used as a type strain for reference. Forty-six MDR clinical isolates of *A. baumannii*, isolated from Los Angeles County hospitals, were used in the antimicrobial susceptibility testing (Table 1). The first batch of 20 outbreak isolates (LAC-1 to LAC-20) were originally described by us in 2008 [14]. Briefly, they were collected from four nosocomial outbreaks from three hospitals in Los Angeles County between 1996 and 2004 by the county Public Health Laboratory (PHL). Isolates LAC-1 through LAC-5 were isolated from an outbreak in Hospital A that lasted for several years (from 1996 to 1999). LAC-6 to LAC-10 were from an outbreak in the same hospital in 2001. The third outbreak was the source of isolates LAC-11 to LAC-15 in Hospital B during 2003–2004, while strains LAC-16 to LAC-20 were archived from an outbreak in Hospital C between 1997 and 1998. The second batch of 26 MDR isolates of *A. baumannii* includes two environmental isolates from the LAC+USC Medical Center and 24 sporadic isolates obtained from mostly adult patients of the medical center (Table 1). There is only one isolate from a 15-year-old female burn patient; the rest of the isolates were obtained from adult patients ranging from 18 to 71 years old. The isolates were obtained from patients in a variety of hospital departments including trauma center/emergency room, operating rooms, intensive care units, burn surgery unit, urology and neurosurgery unit.

Table 1
Bacterial species and strains used.

Species or strains	Description	Source
E. coli K-12 MG1655	Screening target strain	ATCC
E. coli ATCC#25922	Susceptibility reference strain	ATCC
P. aeruginosa ATCC#27853	Susceptibility reference strain	ATCC
E. faecalis ATCC#29212	Susceptibility reference strain	ATCC
S. aureus ATCC#29213	Susceptibility reference strain	ATCC
A. baumannii ATCC#19606	Type strain	ATCC
A. baumannii LAC-1*	Clinical strain isolated in 1999	PHL
A. baumannii LAC-2	Clinical strain isolated in 1996	PHL
A. baumannii LAC-3	Clinical strain isolated in 1996	PHL
A. baumannii LAC-4	Clinical strain isolated in 1997	PHL
A. baumannii LAC-5	Clinical strain isolated in 1997	PHL
A. baumannii LAC-6	Clinical strain isolated in 2001	PHL
A. baumannii LAC-7	Clinical strain isolated in 2001	PHL
A. baumannii LAC-8	Clinical strain isolated in 2001	PHL
A. baumannii LAC-9	Clinical strain isolated in 2001	PHL
A. baumannii LAC-10	Clinical strain isolated in 2001	PHL
A. baumannii LAC-11	Clinical strain isolated in 2003	PHL
A. baumannii LAC-12	Clinical strain isolated in 2003	PHL
A. baumannii LAC-13	Clinical strain isolated in 2003	PHL
A. baumannii LAC-14	Clinical strain isolated in 2004	PHL
A. baumannii LAC-15	Clinical strain isolated in 2004	PHL
A. baumannii LAC-16	Clinical strain isolated in 1997	PHL
A. baumannii LAC-17	Clinical strain isolated in 1997	PHL
A. baumannii LAC-18	Clinical strain isolated in 1997	PHL
A. baumannii LAC-19	Clinical strain isolated in 1997	PHL
A. baumannii LAC-20	Clinical strain isolated in 1998	PHL
A. baumannii LAC-22	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-24	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-25	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-32	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-33	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-35	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-36	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-37	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-38	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-39	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-41	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-50	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-54	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-76	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-79	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-82	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-87	Clinical strain isolated in 2008	LAC+USC
A. baumannii LAC-106	Environmental	LAC+USC
A. baumannii LAC-108	Environmental	LAC+USC
A. baumannii LAC-125	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-135	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-150	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-151	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-153	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-154	Clinical strain isolated in 2009	LAC+USC
A. baumannii LAC-157	Clinical strain isolated in 2009	LAC+USC

LAC+USC, LAC+USC Medical Center; PHL, Public Health Laboratory of the County of Los Angeles.
* LAC: isolates obtained from hospitals located within Los Angeles County as described previously [14].

2.2. HTS

A chemical library of 42,944 small-molecule compounds for screening was purchased from ChemBridge Corp. (San Diego, CA, USA). These compounds were selected from its much larger compound collections for structural diversity and drug-like properties. Reactive, unstable and potentially toxic compounds were eliminated in the selection process. All compounds have molecular weights between 150 and 480 with calculated LogP values (cLogP) below 5.

The 42,944 compounds were distributed on 122 clear polystyrene 384-well plates. Each plate contained 352 different compounds (20 μL, 25 μg/mL, 2.5% dimethyl sulfoxide (DMSO)), 16 positive controls and 16 negative controls. The negative control wells contained no screening compounds, and the positive control wells contained no *E. coli* cells (the seeding of *E. coli* cells was very low, and the reading by OD_{600} was almost the same as medium with no cells). In the HTS, an overnight culture of *E. coli* cells was grown in 5 mL of Luria broth (LB) medium (Fisher Scientific, Tustin, CA, USA). The culture was diluted 100-fold with fresh LB medium and cultured until OD_{600} reached 0.6 for log-phase growth. This culture was further diluted 200-fold with fresh LB medium and dispensed (60 μL per well) to each well of the plates with compounds (final concentration: compound, 6.25 μg/mL; DMSO, 0.625%). The plates were incubated at room temperature for 12–14 h before the absorbance at 600 nm was read on an Envision plate reader (Perkin–Elmer). Statistical variations on each of the plates, expressed as z'-factors [13], were calculated using the readings from positive and negative controls on each plate.

2.3. Hit confirmation by IC_{50} determination

Hit compounds identified from the above-mentioned screening were confirmed by determining IC_{50} values at multiple compound concentrations. The IC_{50} value is the concentration of compound that inhibits bacterial growth by 50%. Using the same proce-

dure as in the primary screening, the growth kinetics were monitored by measuring absorbance continuously at 37°C, instead of end-point reading, using a SpectraMax Plus microplate spectrophotometer (Molecular Device, Sunnyvale, CA, USA) for 8 h. The IC_{50} values were obtained from non-linear curve fitting of the plot of percent inhibition vs. compound concentration [I] by using the equation, % Inhibition $= 100/[1 + (IC_{50}/[I])^k]$, where k is the Hill coefficient.

2.4. Acquisition of additional isothiazolone derivatives

Additional isothiazolone compounds were purchased from commercial vendors (Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific) to assess SAR.

2.5. Antimicrobial susceptibility testing

Minimum inhibitory concentration (MIC) assays using broth microdilution methods were carried out in 96-well microplates based on a modified procedure described previously [14] according to the guidelines of the Clinical and Laboratory Standards Institute [15]. Antibiotic and isothiazolone powders were dissolved in 100% DMSO, 5% DMSO or phosphate-buffered saline (PBS) per manufacturer's instructions and filter sterilized with a 0.2-μm syringe filter (Fisher Scientific, Chino, CA, USA). All compounds and control antibiotics tested were tested from a range of 0.0312 to 256 μg/mL, except Compounds **6** and **7** which were tested from a narrower range of 0.0312–32 μg/mL due to their high potency and their high acquisition cost. Minimum Bactericidal Concentration (MBC) assays were carried out based on Clinical & Laboratory Standards Institute (CLSI) guidelines [16].

2.6. Time-kill studies

Time-kill assays were carried out by the broth method according to the guidelines of the CLSI [16]. Time-kill experiments were performed at least twice. Time-kill curves were generated by GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

2.7. Emergence of resistance

To determine whether resistant mutants can be obtained, representative *A. baumannii* clinical isolates at approximately 5×10^5 colony-forming units (CFU)/mL were plated on to cation-adjusted Mueller–Hinton Agar (MHA) plates containing $2\times$ and $8\times$ MIC of a given isothiazolone compound. Cell inoculum was prepared based on CLSI guidelines [15]. Compounds were serially diluted twofold in 15-mL conical tubes using 5% DMSO as the diluent. One part of the $10\times$ compound dilution was then added to nine parts of the molten MHA previously cooled to 55°C after the medium was removed from the autoclave (to avoid potentially inactivating compounds by high temperature). Bacterial inoculum (20 μL) was applied to the agar surface and spread evenly. After 48 h of incubation, the plates were examined for presence or absence of resistant colonies. The experiments were performed twice, each with duplicates, for a total of four replicates per isolate for each isothiazolone concentration.

2.8. Cytotoxicity Testing

To evaluate the potential toxic effects of the isothiazolone compounds against mammalian cells, cytotoxicity testing was carried out. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA) and phenazine methosulfate (PMS) (Sigma-Aldrich, St. Louis, MO, USA) were used to determine cell viability. Thioridazine hydrochloride (MP Biomedicals, Santa Ana, CA, USA) was used as a positive control compound. HepG2 cells (ATCC HB-8065) in Eagle's Minimum Essential Medium were supplemented with 10% fetal calf serum (Gibco), 10,000 units of penicillin and streptomycin (Gibco), and Glutamax (Gibco). The cells were seeded in a 96-well plate (Costar flat-bottom, polystyrene, tissue culture treated) and incubated overnight at 37°C in the presence of 5% CO_2. After 24 h, isothiazolone compound solutions (10 μL/well) serially diluted in 5% DMSO were added to the designated wells in triplicate. The growth control column received 10 μL of 5% DMSO. After 24 h of incubation at 37°C in the presence of 5% CO_2, 20 μL of MTS/PMS solution was pipetted into each well using a multichannel pipette for a final volume of 120 μL. Plates were read within 10 min of adding the MTS/PMS solution and after 3 h of incubation at 37°C. The absorbance (OD_{490}) was measured using a Tecan Infinite Spectrophotometer (Tecan US, Inc., Morrisville, NC, USA). Assays were performed at least twice with a total of six replicates per compound. The IC_{50} values were obtained from non-linear curve fitting of the plot of percent inhibition versus compound concentration using GraphPad Prism software.

3. Results

3.1. Inhibitors of E. coli growth identified by HTS

Because there is a greater need for novel antibiotics effective against Gram-negative pathogens, we set out to identify inhibitors of *E. coli* cell growth because *E. coli* is a model Gram-negative bacterium. The HTS campaign using *E. coli* MG1655 strain generated 26 compounds with inhibition of cell growth above 50% and 55 compounds above 20% when the concentration of screening compound was 6.25 μg/mL. The z'-factor on the 122 plates ranged from 0.63 to 0.94, with averages of the z'-factors being 0.86 ± 0.05, indicating high-quality data in the HTS. The results of a representative screening plate are shown in Fig. 1.

3.2. Hit confirmation using IC_{50} determination

All 55 hit compounds in the *E. coli* MG1655 screen were further confirmed with IC_{50} determination, with 12 compounds having an IC_{50} lower than 1 μg/mL, 10 compounds with IC_{50} higher than 6.25 μg/mL, and the rest in between. Interestingly, two important classes of compounds and a group of nitro-containing compounds emerged (Fig. 2). One class comprises analogs of quinolones/fluoroquinolones, which include current antibiotics such as ciprofloxacin (Compound **1**, Fig. 2) and a close analog of ciprofloxacin (Compound **2**, Fig. 2). Among the confirmed hit compounds are two additional derivatives of fluoroquinolone (Compounds **3** and **4**, Fig. 2) and a derivative of quinolone (Compound **5**, Fig 2). The second class consists of a derivative of isothiazolone (Compound **6**, Fig 2). An analog of this isothiazolone was identified from the vendor's database (Chembridge Corp), and was obtained for antimicrobial susceptibility testing and subsequent studies (Compound **7**, Fig. 2). Furthermore, a group of compounds possessing nitro groups were also confirmed to be active against *E. coli* MG1655 (Compounds **8**-**18**).

3.3. Antimicrobial susceptibility testing

To evaluate antimicrobial susceptibility of 46 MDR *A. baumannii* clinical isolates [14,17] to the identified inhibitors, MICs were determined for 29 hit compounds of highest potency identified via HTS and one analog of the hit Compound **6**. Results indicated that except for two isothiazolone compounds (Compounds **6** and **7**),

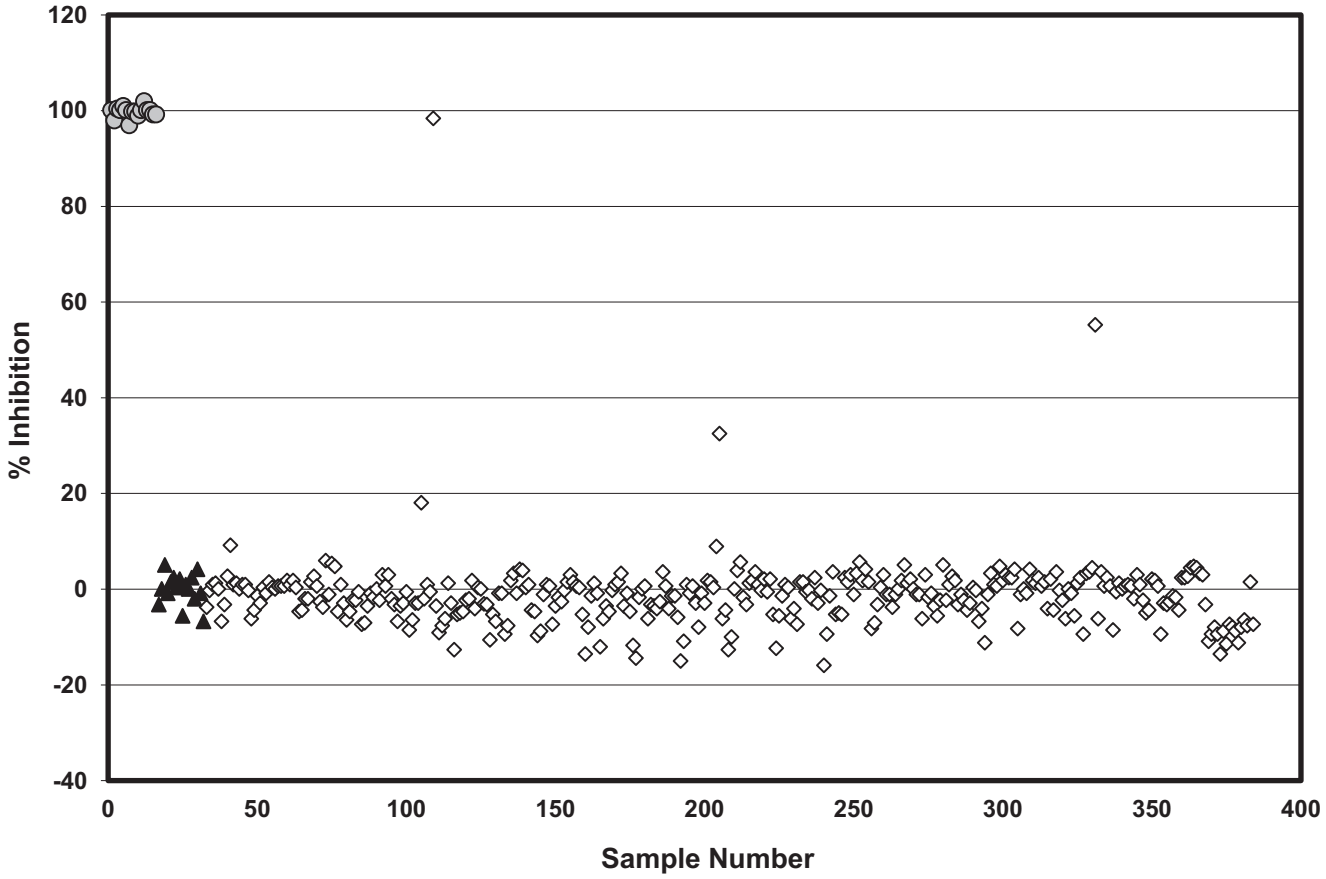


Fig. 1. Typical data from high-throughput screening using *Escherichia coli* MG1655 on one 384-well plate. Positive controls are in wells 1–16 (gray circles), negative controls are in wells 17–32 (solid triangles), and 352 compounds are in wells 33–384 (open diamonds).

most of the hit compounds had little or no activity against these clinical isolates (data not shown) presumably due to the multidrug resistance phenotype of these isolates (e.g., resistance to fluoroquinolones, see below). While Compounds **1** (ciprofloxacin) and **2** (a ciprofloxacin analog) retained activities against *A. baumannii* type strain ATCC#19606 with MICs at 0.5 μg/mL (data not shown), they were inactive against the majority of clinical *A. baumannii* isolates, indicative of a fluoroquinolone resistance phenotype. In fact, antimicrobial susceptibility testing using pure ciprofloxacin indicated that these 46 clinical isolates were indeed classified as resistant to ciprofloxacin [14,17]. While two other fluoroquinolone analogs (Compounds **3** and **4**, Fig. 2) exhibited potent activity against the *E. coli* reference strain ATCC#25922 (data not shown), they had no inhibitory activity against either the clinical isolates of *A. baumannii* or its type strain ATCC#19606 (data not shown). Compounds **6** and **7**, derivatives of isothiazolone, had MIC values of 2 μg/mL or less against these 46 MDR clinical isolates (Supplementary Table S1), with **7** being more potent (MICs at 0.25 μg/mL or less). Additionally, Compounds **6** and **7** were found to be cidal against all 46 MDR clinical isolates of *A. baumannii*, with MBCs matching MIC values (Supplementary Table S1). Moreover, these two compounds (**6** and **7**) were also shown to be active against two Gram-positive reference strains (*S. aureus* ATCC#29213 and *E. faecalis* ATCC#29212) with MICs $\leq$0.5 μg/mL. Surprisingly, Compounds **6** and **7** also possessed inhibitory activity against *P. aeruginosa* ATCC#27853, with MICs of 8 μg/mL and 4 μg/mL, respectively. Although a number of nitro-compounds were confirmed as hits, only some of them exhibited activity against the clinical isolates at 16 or 32 μg/mL (data not shown).

Because two isothiazolone compounds were found to possess exceptional potency against clinical isolates of *A. baumannii*, four additional isothiazolones were acquired to determine SAR (Table 2). The MICs and MBCs of the six isothiazolone compounds were determined against a panel of 46 *A. baumannii* clinical isolates. Compounds **6**, **7**, **21** and **22** exhibited potent and bactericidal activities with MIC and MBC values of 2 μg/mL or less for all 46 *A. baumannii* clinical isolates (Supplementary Table S1), with the exception of three isolates whose MBCs towards **21** are greater than 2 μg/mL. While Compound **20** exhibited potent activity with an MIC of 2 μg/mL against all isolates tested, it did not exert consistent bactericidal effects (Supplementary Table S1). In contrast, Compound **19** had the least activity against the isolates and therefore, is not active. Furthermore, Compounds **6** and **7** were found to be similarly bactericidal against all reference and type strains: *E. coli* (ATCC#25922), *P. aeruginosa* (ATCC#27853), *E. faecalis* (ATCC#29212), *S. aureus* (ATCC#29213) and *A. baumannii* (ATCC#19606) (data not shown). Significantly, the MBC values against *P. aeruginosa* were 16 and 8 μg/mL for Compounds **6** and **7**, respectively (data not shown).

3.4. Time-kill studies

The relationship between the bactericidal activity and the concentration of the isothiazolone compounds was determined through time-kill studies. Four representative *A. baumannii* clinical isolates were selected for these studies: LAC-4, LAC-8, LAC-10, and LAC-13. Compounds **6**, **7**, **21** and **22** were selected for these studies because of their potent MIC and MBC values (Supplementary Table S1). Fig. 3A shows representative time-kill curves for Compound **6**, with polymyxin B (PXB) used as a comparator drug. Maximum killing took place at concentrations 2 $\times$ MIC and higher, with

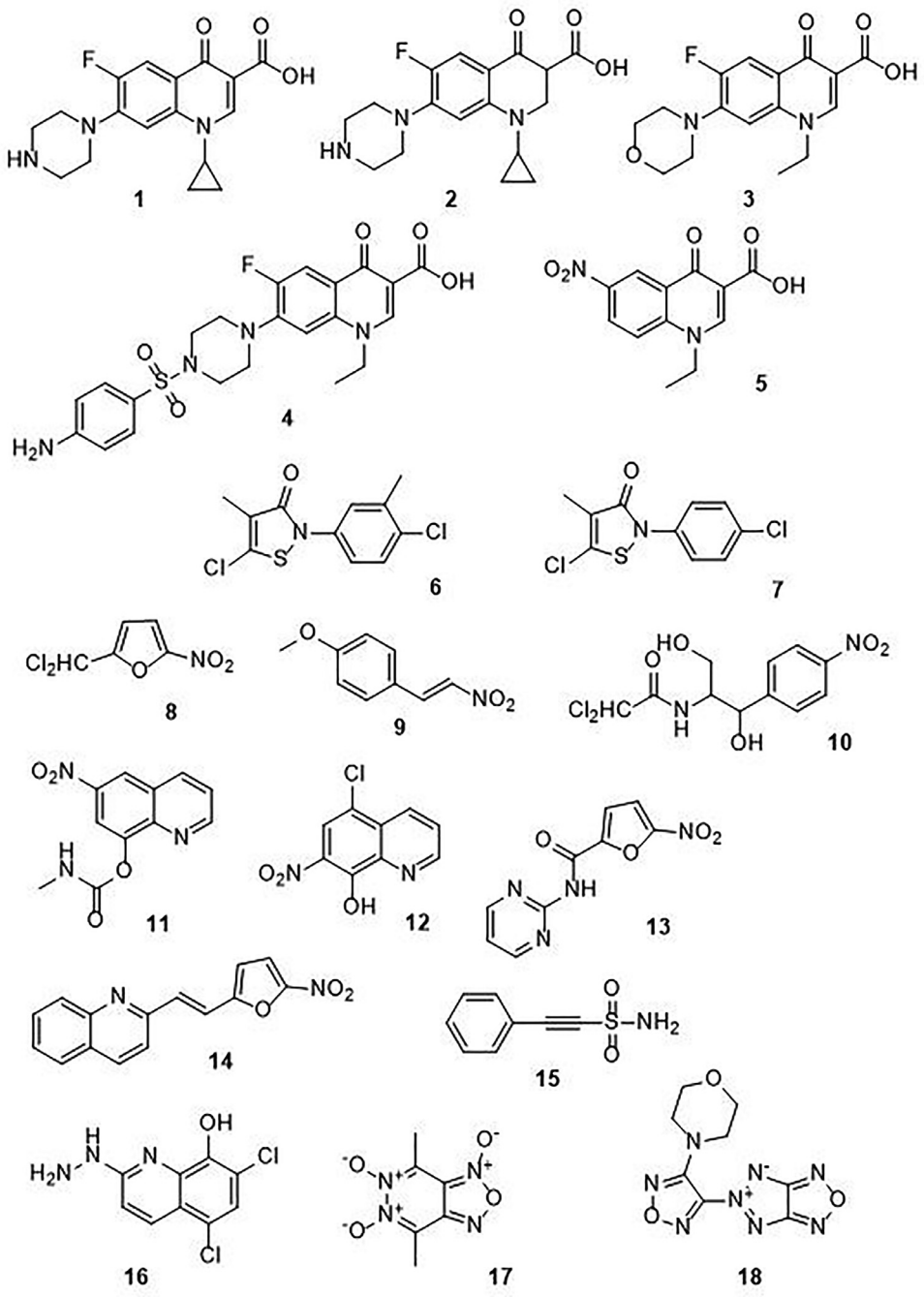


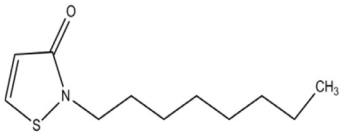
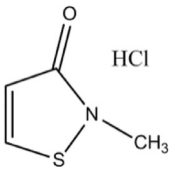
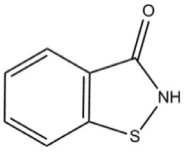
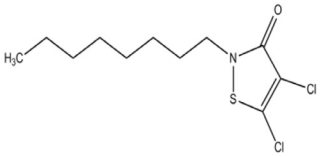
Fig. 2. Structures of select confirmed hits from the screen against *Escherichia coli* MG1655 strain. **1**–**4**, fluoroquinolones; **5**, a quinolone derivative; **6**, 5-chloro-2-(4-chloro-3-methylphenyl)-4-methyl-3(2H)-isothiazolone; **7**, 5-chloro-2-(4-chlorophenyl)-4-methyl-3(2H)-isothiazolone; **8**–**18**, nitro compounds.

a rapid killing (4-log decrease in the number of CFU/mL) occurring within 30 min after compound exposure. Viable counts of LAC-4 cells treated 1 × to 8 × MIC of Compound **7** rapidly dropped about 4-log units after 15–30 min, indicating even faster bactericidal activity of Compound 7 (Fig. 3B). PXB was shown to be bactericidal at 2 × and 8 × MIC. However, unexpectedly, a rebound effect for 2 × MIC PXB was observed after 45 min (Fig. 3A,B). Similar results were observed with LAC-8, LAC-10, and LAC-13 (data not shown).

3.5. Emergence of resistance

Emergence of resistance experiments were carried out to observe whether single step mutation resulting in resistance to a given isothiazolone compound can develop in bacterial cells. Four representative *A. baumannii* clinical isolates were selected for these studies: LAC-4, LAC-8, LAC-10, and LAC-13. Compounds **6**, **7**, **21** and **22** were selected for these studies because of their potent MIC and MBC values (Supplementary Table S1). No resistant mutants of *A. baumannii* isolates were obtained for Compounds **6**, **7** or **22** at compound concentrations 2 × or 8 × MIC. However, for LAC-13 there were resistant mutants for Compound **21** at 2 × MIC (data not shown), which is difficult to interpret. We speculate that perhaps LAC-13's different genetic context [14] from the other three isolates renders an increased frequency for emergence of mutants that are resistant to Compound **21**.

Table 2
Additional isothiazolone compound names and structures.

Compound code	Compound	Chemical structure
19	2-N-Octyl-4-isothiazolin	
20	2-Methyl-4-isothiazolin-3-one-hydrochloride	
21	1,2-Bezisothiazol-3(2H)-one	
22	4,5-Dichloro-2-octyl-isothiazolone	

3.6. Cytotoxicity testing

Cytotoxicity to a mammalian cell line was evaluated to determine the degree to which the six isothiazolone compounds exhibit toxic properties. HepG2 cells were exposed to escalating concentrations of each compound and thioridazine (positive control). Fig. 4 shows dose–response curves and IC_{50} values for the six isothiazolone compounds. Compound **6** displayed the highest cytotoxicity with an IC_{50} of 1.4 μg/ml, while Compound **19** displayed the lowest cytotoxicity with an IC_{50} of 12.3 μg/ml. Compound **7** was less cytotoxic (3.5 μg/ml) than Compound **6**, followed by **21** (4.8 μg/ml), **22** (9.4 μg/ml), and **20** (11 μg/ml). Based on the IC_{50} value and MIC, the therapeutic index can be determined as the ratio of IC_{50} vs. MIC. The therapeutic index describes the potential of a new antimicrobial agent as a future therapeutic. A high therapeutic index is indicative of highly selective for the pathogen of interest and low toxicity to the mammalian cells. Compound **7** had the highest therapeutic index at 28, while Compound **19** had the lowest at 0.048 (Supplementary Table S2).

4. Discussion

The history of antimicrobial therapy has demonstrated that once a new antibiotic is approved for clinical use, resistant strains will emerge sooner or later [9]. Because the majority of antibacterial agents in clinical development are intended for treatment against Gram-positive pathogens, there is an urgent need to restock the clinical pipeline intended for Gram-negative pathogens [18]. In an effort to discover potential antibacterial compounds with activity against Gram-negative pathogens such as *A. baumannii*, we performed antibacterial screens of 42,944 diverse compounds using a nonpathogenic Gram-negative model bacterium, *E. coli* strain MG1655. We identified 55 confirmed active hit compounds, a surprisingly low hit rate (0.0013%) for an HTS, similar to the hit rate (0.0025%) obtained by De La Fuente and colleagues [10]. We speculate that the cell envelope of Gram-negative bacteria might act as a barrier for entry of compounds into bacterial cells. Of the pure compounds tested for antimicrobial susceptibility, two isothiazolones (Compounds **6** and **7**, Fig. 2) exhibited potent activity against a panel of 46 MDR *A. baumannii* isolates (Supplementary Table S1). These compounds along with additional four isothiazolone derivatives were investigated in more detail in this study. The limited number of hit compounds and even fewer compounds with activity against clinical isolates of *A. baumannii* illustrate the tremendous challenges faced in identifying antibacterial compounds, especially against Gram-negative pathogens [19].

Isothiazolones are a class of compounds previously found to possess antibacterial activity and were used traditionally as biocides [20–22]. Khalaj and coworkers synthesized a series of isothiazolone derivatives with and without chloro-substitution at the C-5 position, and tested for antibacterial activity [21]. Most of the compounds exhibited moderate to high activity against both Gram-positive and Gram-negative bacteria. Since the mid-2000s, one series of isothiazolones called isothiazoloquinolones has been investigated by Pucci and colleagues [23,24]. Their current lead drug candidate, ACH-702, is structurally similar to quinolones but differs due to the presence of an isothiazolone ring fused to the quinolone moiety [24]. ACH-702 exhibits broad-spectrum potent activity against both Gram-positive and some Gram-negative bacteria and biochemical assays indicate potent dual inhibition of the essential DNA gyrase and topoisomerase IV [19,24]. More recently, a different structural series of novel isothiazolones was discovered and characterized by Charrier and colleagues [25,26]. Their lead candidate, REDX04957, and its two enantiomers (the *S*-enantiomer REDX05967 and the *R*-enantiomer REDX05990) showed broad-spectrum bactericidal activity against major pathogens, except *Enterococcus* spp. [25,26]. Additionally, these isothiazolone derivatives do not possess a quinolone moiety (unlike ACH-702 which does) but still exert their activity via inhibition of DNA gyrase and topoisomerase IV [25,26]. The two potent isothiazolone inhibitors described in this report, Compounds **6** and **7**, represent a novel series of isothiazolones distinct in structure from the

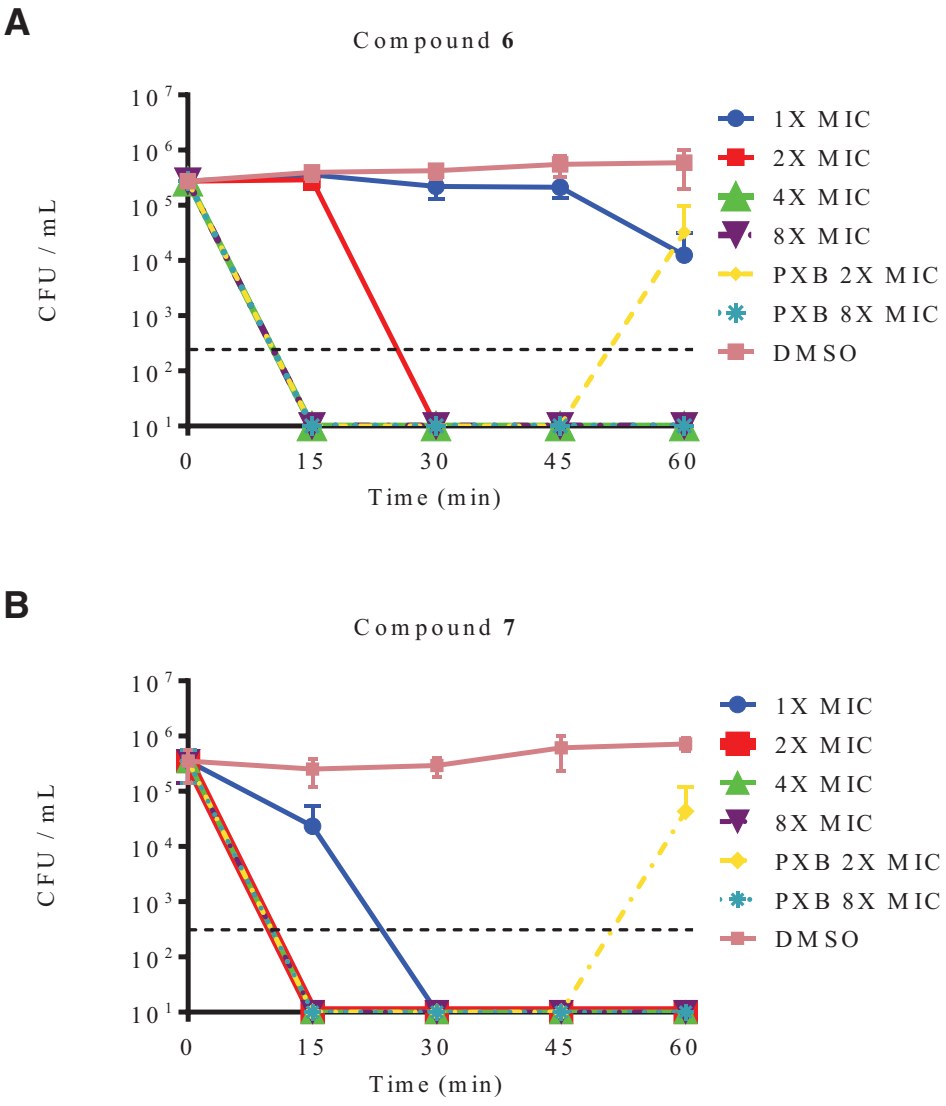


Fig. 3. Time-kill curves of isothiazolone compounds against LAC-4. (A) Comparative activities of Compound **6** at MIC multiples ranging from 1 × to 8 × MIC versus PXB at 2 × and 8 × MIC. (B) Comparative activities of Compound **7** at MIC multiples ranging from 1 × to 8 × MIC versus PXB at 2 × and 8 × MIC. The dashed line represents the bactericidal level. Data are shown as means ± standard deviations. Each graph depicted is representative of at least two experiments.

traditional isothiazolone biocides and the ACH-702 and REDX04957 series of isothiazolone derivatives, and thus warrant detailed investigation.

A. baumannii was named among the top MDR pathogens causing hospital infections worldwide and successfully escaping the effects of antibacterial drugs [1,8]. This pathogen's ability to upregulate innate resistance mechanisms and respond swiftly to environmental pressures rivals those of other Gram-negative pathogens [27]. The antimicrobial susceptibility of the 46 *A. baumannii* clinical isolates obtained from hospitals in Los Angeles County [14] (unpublished results) was evaluated against the six isothiazolone compounds (Figure 2 and Table 2). Of the six isothiazolone compounds, four exhibited potent and bactericidal activities (Compounds **6**, **7**, **21** and **22**). The MICs and MBCs for Compounds **6** and **7** were significantly lower than those of the other four isothiazolones. Compound **20** displayed potency against the panel of clinical isolates, but no consistent bactericidal activity. Compound **19** was inactive against the majority of clinical isolates. These results indicate that the presence of chloro-groups on the heterocyclic ring (Compounds **22**, **6**, and **7**) or a fused benzene ring (Compound **21**) may contribute to the potent and bactericidal antibacterial properties of these compounds, as observed previously [21].

The four isothiazolones that exhibited bactericidal properties during the antimicrobial susceptibility testing were further evaluated in time-kill studies. Compounds **6** and **7** were found to be rapidly bactericidal against a representative *A. baumannii* clinical isolate (Fig. 3A,B). At concentrations at or above 2 × MIC, bacterial cell number was reduced greater than 4 logs for both Compounds **6** and **7** within 30 min, similar to the effect of PXB. However, a cell-count rebound effect can be observed after 45 min with the control drug PXB at 2 × MIC, for unknown reasons. We initially selected PXB as a comparator drug because of its potency displayed against the panel of clinical isolates. Knowledge about the pharmacological properties of PXB is limited and use of this antibiotic was restricted due to its toxicity [28]. The inconsistent activity observed with PXB and the rapid bactericidal properties of Compounds **6** and **7** support continued investigations on this novel series of isothiazolones as a potential therapeutic candidate.

Emergence of resistance experiments allowed us to determine if resistant mutants that overcome the effects of our compounds

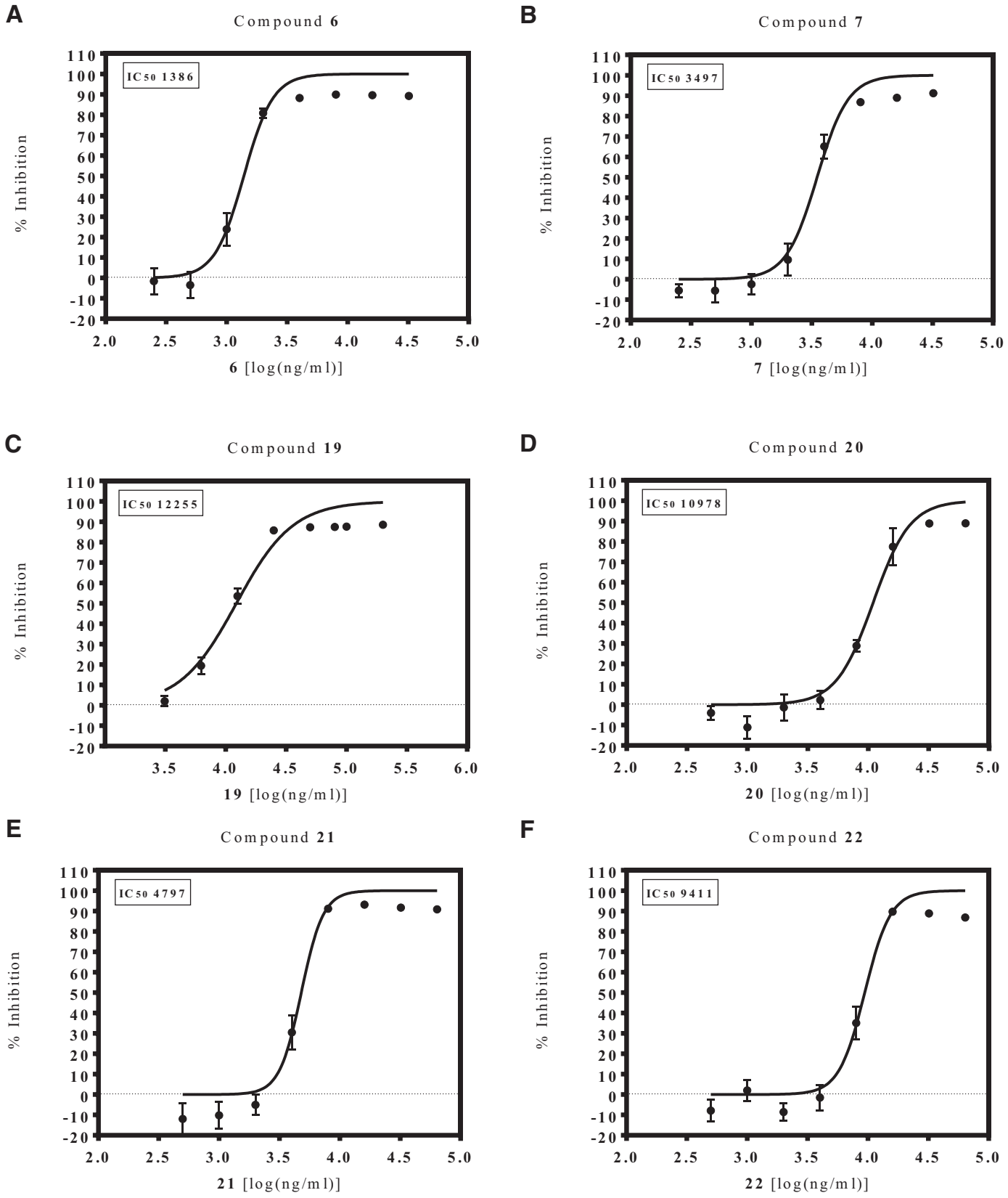


Fig. 4. Cytotoxicity of isothiazolone compounds. (A) Dose–response curve for Compound **6**; (B) dose–response curve for Compound **7**; (C) dose–response curve for Compound **19**; (D) dose–response curve for Compound **20**; (E) dose–response curve for Compound **21**; (F) dose–response curve for Compound **22**. IC_{50} values are shown at the top left corner of each graph. Each graph depicted is representative of at least two experiments.

can emerge. Resistant colonies were only observed for LAC-13 at 2 × MIC for Compound **21**. No resistant colonies grew in the presence of Compounds **6**, **7** or **22** at the concentrations of compounds at MIC multiples (2 ×, 8 × and 16 × MIC). Lack of resistant colonies suggests either a complex mechanism of action or a non-specific mode of action.

Using the human hepatocarcinoma cell line HepG2 as a metabolically active cellular test system, we determined the levels of cytotoxicity these compounds may exert. Of the compounds, **6** exhibited the strongest cytotoxicity, with an IC_{50} of 1.4 μg/mL (Fig. 4A), while Compound **7** was the second, with a slightly higher IC_{50} of 3.5 μg/mL (Fig. 4B). For the isothiazolone compounds tested, we selected the MIC value that appeared most frequently for the clinical isolates during the antimicrobial susceptibility testing (Supplementary Table S1). Of the compounds, **7** has the highest therapeutic index at 28 followed by Compound **6** at 5.5.

Bacterial strains resistant to marketed antimicrobial agents will continue to emerge, thus new antibiotics will continuously be required [19]. The studies reported here further validated the strategy of HTS followed by hit confirmation and in vitro experimental evaluations. Compounds **6** and **7** are novel isothiazolones that have demonstrated consistent potent and bactericidal activities that warrant further investigation. The results from this project will contribute to the discovery and development of novel antibacterial agents to combat MDR bacterial pathogens.

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Competing Interests

None declared.

Ethical approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.12.007.

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