Journal of Global Antimicrobial Resistance 19 (2019) 1–7

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

An aryl isonitrile compound with an improved physicochemical profile that is effective in two mouse models of multidrug-resistant *Staphylococcus aureus* infection

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ARTICLE INFO

Article history:
Received 6 February 2019
Received in revised form 10 April 2019
Accepted 23 April 2019
Available online 30 April 2019

Keywords:
Antimicrobial resistance
Keratinocytes
MRSA
Skin infection
Aryl isonitriles

ABSTRACT

Objectives: The aim of this study was to investigate the antibacterial activity of a synthetic aryl isonitrile compound (**35**) that was developed as part of a compound library to identify new antibacterial agents effective against methicillin-resistant *Staphylococcus aureus* (MRSA).
Methods: Compound **35** was evaluated against MRSA isolates by the broth microdilution assay and for toxicity to mammalian keratinocytes using the MTS assay. A multistep resistance selection assay was conducted to investigate MRSA resistance development to **35**. A Caco-2 bidirectional permeability assay was employed to evaluate the ability of **35** to permeate across the gastrointestinal tract, and compound **35** was incubated with human liver microsomes to determine susceptibility to hepatic metabolism. Finally, compound **35** was evaluated in an uncomplicated MRSA skin infection mouse model and an MRSA neutropenic thigh infection mouse model.
Results: Compound **35** inhibited the growth of MRSA clinical isolates at 2–4 μM and was non-toxic to human keratinocytes. No resistance formation was observed with MRSA against compound **35** after 10 serial passages. In a murine skin wound model, compound **35** significantly reduced the burden of MRSA, similar to the antibiotic fusidic acid. Compound **35** exhibited a marked improvement both in permeability and stability to hepatic metabolism (half-life >11 h) relative to the first-generation lead compound. In a neutropenic thigh infection mouse model, compound **35** successfully reduced the burden of MRSA in immunocompromised mice.
Conclusion: In summary, compound **35** was identified as a new lead aryl isonitrile compound that warrants further investigation as a novel antibacterial agent.

1. Introduction

Bacterial infections resistant to currently available antibiotics continue to pose a major global public-health threat. Among the most challenging pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) remains a frequent cause of community-acquired and healthcare-associated infections. Although enhanced surveillance and prevention programmes have contributed to a decline in infections in hospitals and clinics in certain developed nations, MRSA remains a significant source of skin and soft-tissue infections (SSTIs) and bloodstream infections worldwide, particularly in North America, South America, southern Europe, and countries in Asia and Africa [1–9]. Isolates resistant to key antibiotics used to treat MRSA infections, namely vancomycin and linezolid, have emerged, necessitating the continuous development of new antibacterial agents, including those with unexploited chemical scaffolds or possessing a unique mechanism of action [10–13].

Our research group recently identified aryl isonitriles as a unique class of compounds with anti-MRSA activity [14]. A library consisting of over 40 aryl isonitrile compounds was synthesised

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https://doi.org/10.1016/j.jgar.2019.04.016
2213-7165/

and screened against important clinical MRSA isolates. The lead compound identified from this first-generation series (compound **13**; Fig. 1) was a potent inhibitor of MRSA growth in vitro. However, the poor physicochemical profile (i.e. inability to permeate across the gastrointestinal tract and rapid hepatic metabolism) of the lead compound precluded its evaluation in relevant animal models of MRSA infection. After closer inspection of the aryl isonitrile compound library, one analogue (compound **35**; Fig. 1) was identified with a key structural difference that we hypothesised would permit its evaluation in animal models of MRSA infection. The present study aimed (i) to further investigate the antibacterial activity of compound **35** against MRSA, (ii) to evaluate this compound's physicochemical profile (i.e. permeability and stability to hepatic metabolism) and (iii) to evaluate compound **35** in two murine models of MRSA infection.

2. Materials and methods

2.1. Synthesis of compound *35*

The complete synthetic protocol and chemical characterisation of compound **35** have been described previously [14].

2.2. Bacterial strains and reagents

Clinical isolates of *S. aureus* were obtained through the American Type Culture Collection (ATCC) and the Network of Antimicrobial Resistance in *S. aureus* (NARSA). Antibiotics were purchased commercially and were dissolved in dimethyl sulfoxide (DMSO) for linezolid and rifampicin or in sterile deionised water for vancomycin. Stock solutions (10 mM) were prepared for all antibiotics. Tryptic soy broth (TSB), tryptic soy agar (TSA), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 96-well plates were all purchased from commercial vendors.

2.3. Evaluation of the antibacterial activity of compound *35* and control antibiotics

The minimum inhibitory concentrations (MICs) of compound **35**, linezolid and vancomycin were determined by the broth microdilution assay following Clinical and Laboratory Standards Institute (CLSI) guidelines [15] with the following modifications. Isolates of *S. aureus* were cultured in TSB and were exposed to compound **35**, linezolid or vancomycin, using triplicate samples, in 96-well plates. Plates were incubated aerobically at 37 °C for ≥18 h before MICs were recorded. The MICs reported represent the lowest concentration of each compound/drug necessary to inhibit visual bacterial growth. The minimum bactericidal concentration (MBC) was determined as described previously [16,17].

2.4. Time–kill assays against MRSA

Time–kill assays were conducted as described previously [17,18]. MRSA NRS123 (USA400) cells in log-phase growth were diluted to 2.0×10^6 CFU/mL and were exposed in triplicate to concentrations equivalent to 4 × MIC of compound **35** or linezolid in TSB. Then, 100 μL samples were collected from each treatment regimen after 0, 2, 4, 8, 12 and 24 h of incubation at 37 °C and were subsequently serially diluted in PBS. Bacteria were then transferred to TSA plates and were incubated at 37 °C for ≥18 h before enumerating CFU/mL.

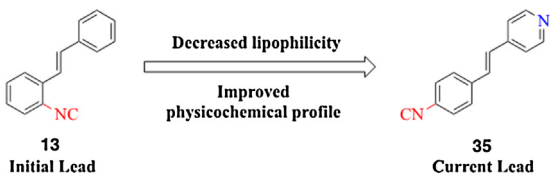

Fig. 1. Chemical structure of compounds **13** and **35**.

2.5. Resistance study for compound *35* against MRSA

A multistep resistance selection experiment was conducted for compound **35** as described previously [17]. The broth microdilution assay was used to determine the MIC of compound **35** and rifampicin (control antibiotic) exposed to MRSA NRS123 (USA400) over 10 passages during a period of 2 weeks. Resistance was classified as a >4-fold increase in the initial MIC, as reported elsewhere [19–22].

2.6. Toxicity assessment against human keratinocytes

Compound **35** was evaluated at concentrations ranging from 16–128 μM against a human keratinocyte (HaCaT) cell line (AddexBio, San Diego, CA, USA) to determine the potential toxic effect to mammalian skin cells in vitro as described previously [23]. In brief, keratinocytes were cultured in DMEM supplemented with 10% FBS at 37 °C with 5% CO_2. Control cells received DMSO alone at a concentration equal to that in compound-treated cell samples. Cells were incubated with compound **35** (using triplicate samples) in a 96-well plate at 37 °C with 5% CO_2 for 24 h. The assay reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for 4 h. The optical density at 490 nm (OD_{490}) was measured using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with compound **35** was expressed as a percentage of the viability of DMSO-treated control cells (mean ± standard deviation of triplicate wells). Toxicity data were analysed by one-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons test ($P < 0.05$) using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

2.7. Evaluation of compound *35* in a murine model of MRSA skin infection

All animal studies were conducted under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and were carried out in strict accordance with the recommendations in the '*Guide for the care and use of laboratory animals*' of the National Institutes of Health (NIH). The mouse study emulated the method described in previous reports with slight modifications [23–28]. Three groups ($n = 5$) of 8-week-old female BALB/c mice (Envigo, Indianapolis, IN, USA) were used. Mice received an intradermal injection (40 μL) containing 1.32×10^9 CFU/mL MRSA USA300 to induce formation of an open wound/abscess. Then, 2 days after infection (once an abscess had appeared in all mice), topical treatment was initiated, with each group of mice receiving a 2% suspension (formulated in petroleum jelly) of either fusidic acid or compound **35**. One group of mice was treated with the vehicle alone (negative control). Each group of mice was housed separately in a ventilated cage with appropriate bedding, food and water. Mice were checked at least four times daily during infection and treatment to monitor adverse reactions. Mice were treated twice daily for 5 days before they were humanely euthanised via CO_2 asphyxiation 12 h following administration of the last dose. Wounds were aseptically extracted and were subsequently homogenised in 2 mL of PBS. The homogenate was serially diluted in PBS before plating onto mannitol salt agar (MSA) plates. Plates were incubated for ≥16 h at 37 °C before viable CFU were counted,

and the MRSA reduction in the skin wound (relative to the negative control) post-treatment was determined for each group.

2.8. Caco-2 bidirectional permeability evaluation of compound **35**

The Caco-2 bidirectional permeability assay was conducted as described previously [14,29].

2.9. Metabolic stability analysis of compound **35**

To investigate the stability of compound **35** to hepatic metabolism, the compound was evaluated with pooled human liver microsomes as described previously [29].

2.10. Investigation of compound **35** in a neutropenic murine MRSA thigh infection model

All animal studies were conducted under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and were carried out in strict accordance with the recommendations in the '*Guide for the care and use of laboratory animals*' of the NIH. Female 6–8-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) weighing 19–20 g were used in this study. The mouse study was conducted similar to a previously published report [30]. All mice were rendered neutropenic via two intraperitoneal (i.p.) injections of 150 mg/kg body weight and 100 mg/kg body weight cyclophosphamide 4 days and 1 day pre-infection, respectively. To initiate infection, the right thigh of the mouse was injected with a 100 μL aliquot of MRSA USA300 (5.35×10^7 CFU/mL). Groups of mice ($n = 5$) were treated 2 h and 12 h post-infection with a 20 mg/kg i.p. injection of compound **35** or 20 mg/kg linezolid. Mice receiving a single i.p. injection of the vehicle at 2 h post-infection served as the negative control group. Three mice were humanely euthanised via CO_2 asphyxiation at 3 h post-infection to enumerate the bacterial load in infected thighs (1.29×10^6 CFU). The remaining groups of mice were humanely euthanised via CO_2 asphyxiation at 24 h post-infection. The right thigh muscle was harvested aseptically, weighed and homogenised in PBS. To determine the bacterial load in the infected thighs post-treatment, the homogenate was serially diluted in PBS and aliquots of each dilution were plated on MSA plates. The plates were incubated for ≥18 h at 37 °C before MRSA colonies were enumerated and compared with the negative control (vehicle alone) group.

3. Results

3.1. Evaluation of compound **35** against strains of drug-resistant S. aureus

Compound **35** was previously found to inhibit the growth of *S. aureus* clinical isolates at concentrations ranging from 2–8 μM [14]. In the current study, the antibacterial activity of compound **35** against two additional MRSA isolates (USA300 and USA400) responsible for the majority of MRSA-related SSTIs in North America [5,31–33] was evaluated. Compound **35** inhibited the growth of both strains at a concentration of 4 μM (USA300) and 8 μM (USA400). This was similar to the activity of compound **35** against two methicillin-susceptible *S. aureus* strains (MICs of 4–8 μM). The antibacterial activity of compound **35** was further investigated against clinical isolates of *S. aureus* resistant to linezolid (NRS119) and vancomycin (VRS4, VRS10 and VRS11a), two antibiotics frequently used to treat MRSA infections. Compound **35** demonstrated a lack of cross-resistance with existing MRSA therapeutics and potent activity against multidrug-resistant clinical isolates. Compound **35** (MIC = 2 μM) was more potent than linezolid (MIC = 32 μM) against MRSA NRS119. Furthermore, compound **35** (MIC = 8 μM) was more effective than vancomycin (MIC > 64 μM) against the three clinical isolates of vancomycin-resistant *S. aureus*.

Table 1
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of compound **35** and control antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA), linezolid-resistant *S. aureus* (LRSA) and vancomycin-resistant *S. aureus* (VRSA) strains.

Strain	Compound **35**		Linezolid		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 6538	8	>64	2	>64	1	2
S. aureus NRS107	4	64	2	16	1	1
MRSA NRS119 (LRSA)	2	4	32	64	1	1
MRSA NRS123 (USA400)	8	>64	1	16	1	1
MRSA NRS384 (USA300)	4	>64	2	16	1	1
VRS4 (VRSA)	8	>64	4	64	>64	>64
VRS10 (VRSA)	8	>64	2	32	>64	>64
VRS11a (VRSA)	8	16	2	32	>64	>64

3.2. Compound **35** exhibits bacteriostatic activity against MRSA

To determine whether compound **35** is a bacteriostatic or bactericidal agent against *S. aureus* in vitro, the MBC was determined. The MBC was found to be ≥64 μM against six of the eight *S. aureus* strains tested (Table 1). The MBCs were >3-fold higher than the MICs for these six *S. aureus* strains, indicating that compound **35** is a bacteriostatic molecule. A similar result was observed for linezolid, an antibiotic previously shown to exhibit bacteriostatic activity against MRSA in vitro [34–36]. To confirm that compound **35** is a bacteriostatic agent against MRSA in vitro, a time–kill assay was conducted against MRSA NRS123 (USA400). A 3-log decrease in MRSA CFU within 24 h would be indicative of bactericidal activity. As depicted in Fig. 2, no decrease in MRSA CFU was observed in the presence of 4 × MIC of compound **35** over 24 h, confirming the compound is bacteriostatic in vitro. Linezolid at 4 × MIC was able to decrease the MRSA CFU by 1.89-$\log_{10}$ over 24 h, confirming its bacteriostatic activity in vitro, similar to previously published studies [34–36].

3.3. MRSA resistance to compound **35** does not emerge after multiple passages

After confirming the antibacterial activity of compound **35** against MRSA, we next investigated whether resistance to this compound would emerge rapidly. A multistep resistance selection experiment was conducted for compound **35** and rifampicin against MRSA USA400 (Fig. 3). After 10 passages, no increase in the MIC of compound **35** was observed, indicating that rapid resistance

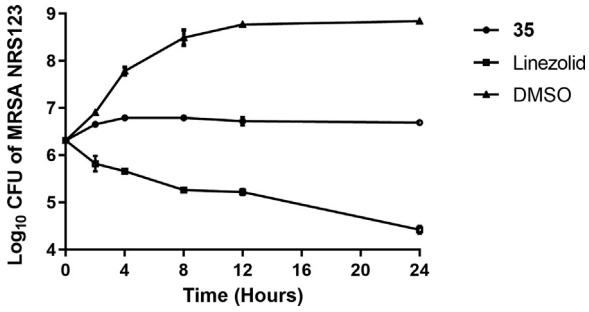

Fig. 2. Time–kill analysis of compound **35** and linezolid at 4× MIC against methicillin-resistant *Staphylococcus aureus* (MRSA) NRS123 (USA400) over a 24-h incubation period at 37 °C. Dimethyl sulfoxide (DMSO) served as a negative control. Error bars represent the standard deviation obtained from triplicate samples used for each compound/antibiotic studied. MIC, minimum inhibitory concentration.

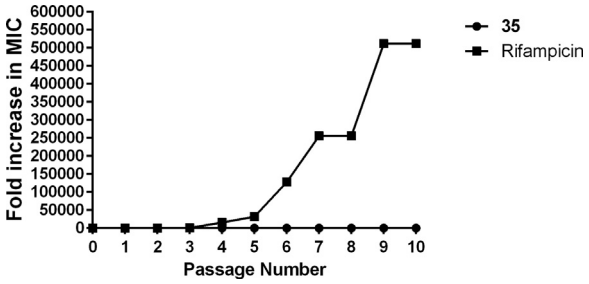

Fig. 3. Multistep resistance selection of compound **35** and rifampicin against methicillin-resistant *Staphylococcus aureus* (MRSA) NRS123 (USA400). MRSA was serially passaged daily over a 10-day period and the minimum inhibitory concentrations (MICs) of compound **35** and rifampicin (control antibiotic) were determined by the broth microdilution assay after each successive passage. A >4-fold increase in MIC would be indicative of bacterial resistance development to the test agent.

of MRSA to compound **35** is unlikely to occur. In contrast, the MIC of rifampicin increased rapidly. By the second passage, the MIC of rifampicin increased nearly 500-fold relative to the antibiotic's initial MIC. The rapid emergence of resistance to rifampicin is in agreement with a previous published report [37].

3.4. Compound **35** is safe to mammalian keratinocytes

S. aureus is a leading source of SSTIs globally [1,3,5–7]. As such, we decided to investigate the antibacterial activity of aryl isonitrile **35** in an MRSA murine skin infection mouse model. Prior to exposing mice to the compounds, the safety profile of keratinocytes (HaCaT) exposed to different concentrations of compound **35** was tested. At the maximum concentration evaluated (128 μM), compound **35** was observed to be safe to keratinocytes, with >90% of HaCaT cells remaining viable (Fig. 4).

3.5. Compound **35** reduces the burden of MRSA in a murine skin infection model

After confirming that compound **35** is non-toxic to keratinocytes, the antibacterial activity of the compound was investigated

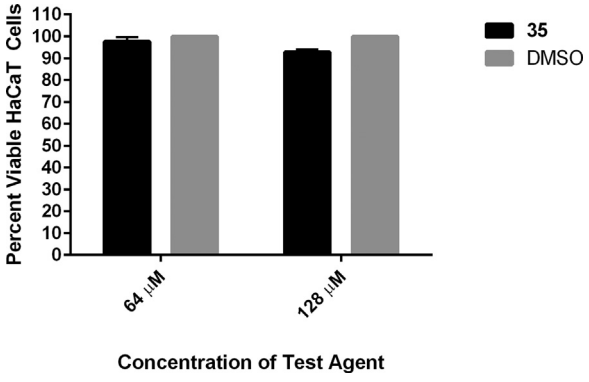

Fig. 4. Toxicity analysis of compound **35** against human keratinocytes (HaCaT). Percent viable mammalian cells, measured as mean OD_{490} ratio of test agent relative to DMSO, for cytotoxicity analysis of compound **35** (tested in triplicate) at 64 μM and 128 μM against HaCaT cells over a 24-h period using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] assay. DMSO was used as a negative control to determine a baseline measurement. OD_{490} values represent the mean of a minimum of three samples analysed for each test agent. Error bars represent the standard deviation of the OD_{490} values. One-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons test determined no statistical difference between the values obtained for compound **35** and DMSO. OD_{490}, optical density at 490 nm; DMSO, dimethyl sulfoxide.

in a mouse model of MRSA skin infection. MRSA USA300 was selected as the infectious agent as this particular strain is the most frequently isolated MRSA strain from SSTIs in the USA [5,32]. After the formation of an abscess at the site of infection, mice were treated twice daily for 5 days. As the skin wounds were uncomplicated and localised, treatment was administered topically directly onto the surface of the abscess. Mice were euthanised 12 h after administration of the last dose and the abscesses were harvested to enumerate MRSA CFU. As shown in Fig. 5, compound **35** (74.10% reduction) was as effective as the control antibiotic fusidic acid (77.78% reduction) in reducing the burden of MRSA in the abscesses of infected mice after only 5 days of treatment. No excess inflammation (redness or swelling around the wound site) or toxicity was observed in wounds after exposure to compound **35** or fusidic acid.

3.6. Assessment of the permeability and metabolic stability profile of compound **35**

The ability of compound **35** to successfully reduce the burden of MRSA in a localised murine skin infection model led us to next investigate the effectiveness of this compound in treating a systemic MRSA infection. Prior to investigating the efficacy of compound **35** in a systemic MRSA mouse model, the ability of the compound to permeate across the gastrointestinal tract and its stability to hepatic metabolism were evaluated. To simulate the ability of **35** to cross the gastrointestinal tract, the compound was evaluated in a Caco-2 bidirectional permeability assay (Table 2). The compound rapidly permeated across the Caco-2 monolayer from the apical to basolateral compartment, with a mean apparent permeability rate of 36.20×10^{-6} cm/s, similar to the high permeability of the control drug propranolol (mean apparent permeability rate of 46.50×10^{-6} cm/s). The low efflux ratio (0.33) observed for **35** indicates that the compound most likely is not a substrate for P-glycoprotein, a major source of efflux of compounds/drugs from the gastrointestinal tract.

Next we assessed the stability of **35** to hepatic metabolism by incubating the compound with pooled human liver microsomes (Table 3). Compound **35** was slowly cleared by hepatic microsomes (mean intrinsic rate of clearance <115.50 μL/min/mg), resulting in an excellent half-life of >11 h. This exceeded the result obtained for two of the positive control drugs, propranolol (>2 h) and imipramine (>2 h). The long half-life observed for compound **35**

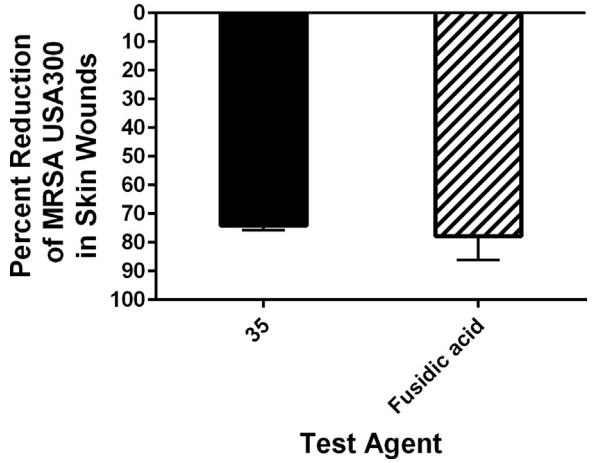

Fig. 5. Reduction of methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 in infected lesions of mice. Mean percent reduction of MRSA CFU/mL in murine skin lesions after treatment with compound **35** or fusidic acid. One-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons test found no statistical difference between mice treated with fusidic acid or compound **35**.

Table 2
Caco-2 bidirectional permeability analysis for compound **35** and control drugs.

Test compound	Mean A→B P_{app} (10^{-6} cm/s)	Mean B→A P_{app} (10^{-6} cm/s)	Efflux ratio[a]	Notes
35	36.20	12.00	0.33	High permeability
Colchicine	0.20	4.90	24.50	P-gp substrate
Ranitidine	0.40	2.00	5.00	Poor-permeability control
Labetolol	17.40	41.40	2.38	Moderate-permeability control
Propranolol	46.50	59.30	1.28	High-permeability control

P_{app}, apparent permeability rate; P-gp, P-glycoprotein.
[a] Efflux ratio = P_{app} (B→A)/P_{app} (A→B).

suggests that once-daily dosing may be a viable option for treatment of systemic MRSA infections.

3.7. Compound **35** successfully reduces the burden of MRSA in a neutropenic thigh infection mouse model

Compound **35** was next evaluated in an MRSA neutropenic thigh infection mouse model. Mice were infected with MRSA USA300 and were subsequently treated with 20 mg/kg of either compound **35** or linezolid. At 1 day post-infection, mice were humanely euthanised and the infected thighs were harvested to enumerate MRSA CFU. As shown in Fig. 6, compound **35** produced a statistically significant reduction in MRSA USA300 (75.44% reduction) compared with mice receiving the vehicle alone. Linezolid reduced the burden of MRSA CFU by 91.7% at the same test concentration (20 mg/kg).

Table 3
Metabolic stability evaluation for compound **35** and control drugs in human liver microsomes.

Test compound	Mean CL_{int} (μL/min/mg)	Mean $t_{1/2}$ (min)	Notes
35	<115.50	660.50	Stable to hepatic metabolism
Terfenadine	752.20	9.25	High-clearance control
Verapamil	352.20	20	High-clearance control
Propranolol	<115.50	131.50	Low-clearance control
Imipramine	<115.50	129.90	Low-clearance control

CL_{int}, intrinsic rate of clearance; $t_{1/2}$, half-life.

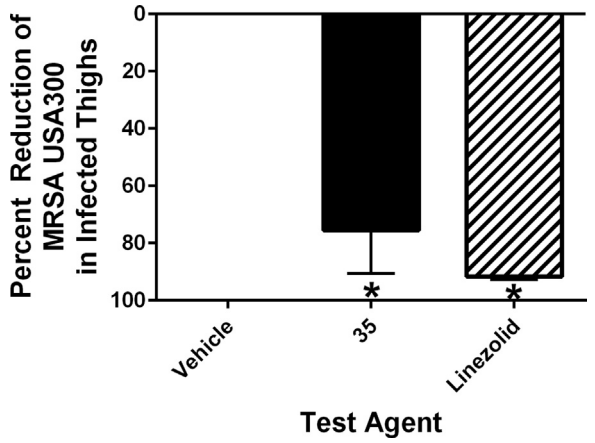

Fig. 6. Reduction of methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 in infected thighs of mice after treatment with compound **35** or linezolid at 20 mg/kg. Mean percent reduction of MRSA USA300 in murine right thighs. One-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons test found a statistically significant difference for mice treated with compound **35** or linezolid (*$P < 0.05$) compared with mice receiving the vehicle (10% dimethyl sulfoxide, 10% Tween 80, 80% phosphate-buffered saline) alone.

4. Discussion

The rapid emergence of bacterial resistance to antibiotics necessitates continuous efforts to discover and develop new antibacterial agents. At present, most antibacterial development initiatives focus on modifying existing drug classes to address the limitations of currently available clinical molecules (i.e. improving potency, expanding the spectrum of activity, mitigating host toxicity or enhancing the pharmacokinetic profile). Although this approach has successfully yielded new antibacterials, these agents are often susceptible to the same resistance mechanisms as previous members of their drug class. Thus, there is a need to identify and develop antibacterial agents with unexploited chemical scaffolds. One of these unique unexplored scaffolds is the aryl isonitrile scaffold. Isonitrile-bearing compounds have previously been found to possess anti-infective activity and have been investigated for use as antimalarial and antifungal agents [38–40]. However, limited investigations have been conducted into aryl isonitriles, particularly synthetic molecules, for use as antibacterial agents.

We previously synthesised and evaluated a series of more than 40 novel compounds bearing the aryl isonitrile scaffold for antibacterial activity against MRSA [14]. These first-generation aryl isonitrile compounds inhibited the growth of MRSA at concentrations ranging from 2–64 μM [14]. However, the lead compound (**13**) from the first-generation series exhibited a poor physicochemical profile that precluded its evaluation in suitable animal models of MRSA infection. This finding is not unexpected as many early-stage lead compounds tend to exhibit a poor physicochemical profile and require further optimisation [41]. It has been observed that compounds with a log*P* ranging from 1–4 tend to exhibit optimal physicochemical properties, particularly for drugs administered systemically [41]. We suspected that the stilbene backbone in **13** (log*P* = 4.22) resulted in a highly lipophilic molecule susceptible to hepatic metabolism. Due to the fact that their binding site is lipophilic, enzymes responsible for metabolism tend to bind more tightly to lipophilic compounds. Substitution of a benzene ring with a pyridine group is one strategy that has been employed by medicinal chemists to improve the stability of a molecule to hepatic metabolism by decreasing the molecule's lipophilicity [42]. We re-visited the aryl isonitrile compound library synthesised and found a second compound (**35**) that exhibited similar potency to the lead compound. Closer inspection of the chemical structures of compounds **13** and **35** revealed two structural differences. First, a replacement of the second benzene ring in **13** with pyridine in compound **35** (log*P* = 3.16), and the ortho isonitrile functionality in **13** showing up in the para position of the isonitrile-bearing aromatic ring of **35**. The para positioning in the isonitrile group in compound **35** can be seen to confer a degree of symmetry and planarity to the compound compared with **13**. Compound **35** was subsequently evaluated for stability to hepatic metabolism by incubating the compound with human liver microsomes.

As anticipated, a noticeable improvement in metabolic stability for compound **35** was observed. Compound **13** was previously found to exhibit a half-life of <1 h [14]. In contrast, compound **35** exhibited a significantly improved half-life of >11 h, which may permit once-daily dosing, which is ideal for patient compliance. It is important to note that the benzene to pyridine substitution can result in a loss of biological activity given that lipophilic groups play an important role in binding to the molecular target [42]. However, no loss of in vitro antibacterial activity for **35** (relative to compound **13**) was observed against MRSA. In addition to the improvement in metabolic stability observed for compound **35**, there was a significant improvement in the molecule's ability to permeate across the gastrointestinal tract. Previously, compound **13** was unable to cross the gastrointestinal tract as simulated by the Caco-2 bidirectional permeability assay (mean apparent permeability rate of 0.0×10^{-6} cm/s) [14]. Remarkably, **35** exhibited a pronounced improvement in the ability to permeate across the Caco-2 bilayer (mean apparent permeability rate from the apical to basolateral compartment of 36.20×10^{-6} cm/s), suggesting that oral dosing of this molecule may be possible.

Compound **35** possesses additional characteristics that make it a promising therapeutic agent, including potent in vitro activity against drug-resistant *S. aureus* isolates and lack of toxicity to mammalian cells (non-toxic to human keratinocytes at 128 μM, i.e. >30-fold higher than the concentration where the compound inhibits MRSA growth in vitro). Furthermore, when investigated in a multistep resistance selection experiment, no MRSA mutants exhibiting resistance to **35** were isolated even after 10 passages. This suggests that repeated exposure/dosing to this compound is unlikely to induce resistant MRSA mutants to emerge rapidly. These features, along with the noticeable improvement in permeability and stability to hepatic metabolism of compound **35**, drove us to investigate this compound in two mouse models of MRSA infection.

As noted in the introduction, MRSA continues to represent a major cause of nosocomial and community-acquired bacterial infections, including SSTIs and bloodstream infections, throughout the world [1,3–7,9]. Strains of MRSA often exhibit resistance to more than one antibiotic, making treatment of MRSA infections challenging [5]. MRSA USA300 is the predominant strain linked to SSTIs in the USA, particularly in the community setting [5,32]. As such, we moved to evaluate the efficacy of compound **35** administered topically in a MRSA skin wound mouse model. Compound **35** significantly reduced the burden of MRSA USA300 in infected abscesses of mice by >70% after only 5 days of treatment in a manner similar to fusidic acid, an agent used topically in Europe to treat MRSA skin infections [43]. Extensive usage of fusidic acid has resulted in the emergence of resistant isolates, necessitating the discovery of alternative agents that can be used to treat uncomplicated MRSA SSTIs [44].

Buoyed by the positive improvement in the physicochemical profile observed with **35**, we moved to evaluate this compound in a MRSA neutropenic thigh infection mouse model. This model evaluates the efficacy of an antibacterial agent in the absence of the host's innate immune response and exhibits good translatability to the efficacy of a drug/compound in humans. After only two doses of compound **35** at 20 mg/kg, a significant reduction (75.4% reduction) in MRSA CFU was observed in the infected thighs of mice compared with mice receiving the vehicle alone. This was lower than the result observed for the control antibiotic linezolid (91.7% reduction) at the same test concentration.

We hypothesised that the difference in effectiveness in vivo between compound **35** and linezolid in the murine thigh infection model may be due to binding to serum proteins, thus decreasing the free fraction of compound available in the circulation. This would necessitate increasing the size or frequency of doses administered to account for the reduced free fraction of compound in circulation. The MICs of compound **35** and linezolid were determined against MRSA USA300 by the broth microdilution assay in the presence and absence of a physiological concentration (4%) of human serum albumin (HSA), the major protein component present in serum (Supplementary material) [45]. The MIC of compound **35** against MRSA USA300 increased from 1 μg/mL to 8 μg/mL in the presence of HSA. Although this increase may partially interfere with the antibacterial activity of compound **35** if administered systemically, using a higher concentration of the compound could potentially resolve this issue. No increase in the MIC of linezolid was observed in the presence of HSA, in agreement with a previous report [45]. A limitation of both the MRSA skin infection and thigh infection mouse models is the number of mice used in each study. Future studies will look to increase the number of mice evaluated, test a broader range of doses/concentrations for compound **35**, and include both male and female mice in order to validate efficacy in a more diverse patient population.

In conclusion, the present study identified an aryl isonitrile compound (**35**) bearing potent antibacterial activity against MRSA in vitro. Compound **35** emerged as a new lead compound based upon its potency against MRSA, safety to mammalian cells, lack of MRSA resistance development and enhanced physicochemical profile. This compound significantly reduced the burden of MRSA both in a murine skin infection model and a neutropenic thigh infection model. However, compound **35** appears to bind to HSA, which slightly reduced its effectiveness compared with linezolid when administered systemically. Addressing this limitation and deducing the molecular target are necessary components to resolve in order to further develop aryl isonitrile compounds as a novel class of antibacterial agents to treat drug-resistant *S. aureus* infections.

Funding

MD has received financial support from the National Institutes of Health (NIH) [R35 GM128570]. The NIH is acknowledged for supporting shared NMR resources to Purdue Center for Cancer Research (West Lafayette, IN, USA) [grant P30 CA023168].

Competing interests

MD has received unrestricted grants from Eli Lilly and Amgen. All other authors declare no competing interests.

Ethical approval

All animal studies were conducted under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) [protocol no. 1207000676] and were carried out in strict accordance with the recommendations in the '*Guide for the care and use of laboratory animals*' of the NIH.

Acknowledgements

The authors would like to thank the Network of Antimicrobial Resistance in *S. aureus* (NARSA) program supported under NIAID/NIH Contract # HHSN272200700055C for providing the MRSA and vancomycin-resistant *S. aureus* (VRSA) strains used in this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.04.016.

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