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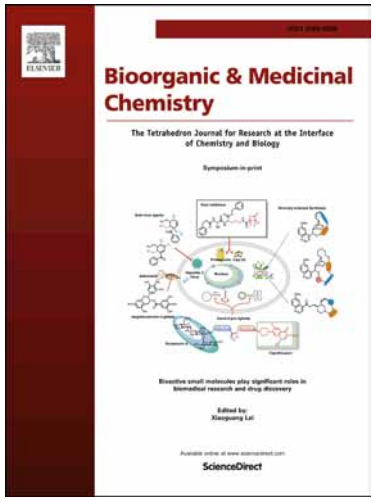

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Novel pyridyl nitrofuranyl isoxazolines show antibacterial activity against multiple drug resistant *Staphylococcus* species.

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Abstract

A novel series of pyridyl nitrofuranyl isoxazolines were synthesized and evaluated for their antibacterial activity against multiple drug resistant (MDR) *Staphylococcus* strains. Compounds with piperazine linker between the pyridyl group and isoxazoline ring showed better activity when compared to compounds without the piperazine linker. 3-pyridyl nitrofuranyl isoxazoline with a piperazine linker was found to be more active than corresponding 2-and 4-pyridyl analogues with MICs in the range of 4-32 μg/mL against MDR *Staphylococcus* strains. The eukaryotic toxicity of the compounds was tested by MTT assay and were found to be non-toxic against both non-tumour lung fibroblast WI-38 and cervical cancer cell line HeLa. The most active pyridyl nitrofuranyl isoxazoline compound showed improved activity against a panel *Staphylococcus* strains compared to nitrofuran group containing antibiotic nitrofurantoin.

Key words: Antimicrobial Resistance, Nitrofuran Isoxazoline, MRSA, Medicinal Chemistry, Antibacterial Activity, Structure Activity Relationship

1.0 Introduction

The development of novel antibacterial agents that are capable of killing resistant bacteria is urgent due to the challenges posed by a number of, both Gram-positive and Gram-negative, pathogens with multi- and sometimes pan-drug resistance [1-4]. An acronym, ESKAPEE has been derived from the organisms recognised as the major threats (*Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp* and *Escherichia coli*) although there are a number of other organisms that may become equally challenging to treat in the clinic [2-7]. The pipeline of antibiotics is essentially empty and very few compounds are in the early stages of clinical trials [2, 3, 8-10]. Of the Gram-positive pathogens, increasing resistance in *S. aureus* has become a major clinical problem, particularly in the hospital environment, causing significant morbidity and mortality in both healthy hosts and those with underlying comorbidities [11-13]. *S. aureus* is found commensally on nasal passages, skin and mucous membranes. The emergence of resistant strains of *S. aureus* has been reported since the mid-1900s. More recently MRSA has become the number one cause of hospital-associated infections, and a large proportion of these infections are caused by MRSA [14]. Approximately 150,000 infections are caused by MRSA in the Europe and the mortality rate is estimated between 15-25%[15] . Therefore, development of novel antimicrobial agents for the treatment of infections caused by MDR *S. aureus* is an urgent priority.

Classical antimicrobial drug discovery involves *in vitro* screening for antimicrobial candidates, Structure Activity Relationship (SAR) analysis, followed by *in vivo* testing for toxicity and efficacy. However, the high cost of drug development, coupled with a relatively short window of use to gain a return on investment for new antibiotics, have resulted in a waning interest in

antibiotic discovery among many pharmaceutical companies[16]. Lack of novel chemical scaffolds with antibiotic activity has further compounded the problem[2], and no new classes of antibiotics have been found since daptomycin, leading to a discovery void[17]. One of the key approaches in discovering new antibacterial drugs is the modification of existing chemical scaffolds, almost all the antibacterial drugs that reached the market after the 1960s are synthetic derivatives of core-scaffolds discovered between 1930 and 1950. Modifications and introductions of novel chemical moieties on the core-scaffold resulted in new generations of molecules typically characterized by greater potency, a broader spectrum of activity and capability of avoiding the mechanism of resistance[2]. In a bid to identify a new chemical scaffold with activity against MDR Gram-positive bacteria, we explored nitrofuran-isoxazoline scaffold that has been previously reported for notable activity against *Mycobacterium tuberculosis*[18-21]. Incorporation of a pyridyl side chain with a piperazine spacer has resulted in compounds (**Fig 1a**) with significant activity against MDR *Staphylococcus* species including MRSA strains. Interestingly, the position of the nitrogen atom in the pyridine ring appears to be very important for antibacterial activity with 3-pyridyl analogue showed the most notable activity followed by the 2-pyridyl analogue while the 4-pyridyl analogue is either inactive or active only at a very high concentration. Incorporation of electron withdrawing or donating groups in the terminal pyridine ring retained activity of the 3-pyridyl compounds. The piperazine spacer appeared to have a notable effect on the antimicrobial efficacy as compounds without the piperazine spacers (**Fig 1b**) were found to be less active compared to their counterparts with piperazine spacers, except the 4-pyridyl analogues which showed some gain of activity after the removal of the piperazine spacer. The activity range of the eight compounds synthesized

provides SAR information about this modified chemical scaffold and its antimicrobial profile that can be further explored to develop more potent analogues.

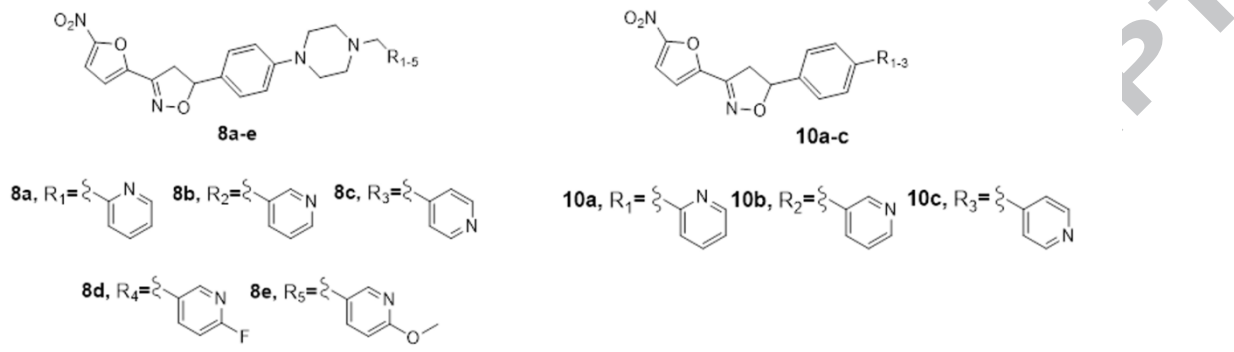

Fig 1. Structures of isomeric pyridyl nitrofuran isoxazolines with a piperazine spacer, 8a-e, and without a piperazine spacer, 10a-c.

2.0 Materials and Methods

2.1 Chemistry

All solvents and reagents for the synthesis were obtained from commercial available sources including Sigma-Aldrich, Fisher Scientific, Fluorochem and Alfa Aesar. Thin-layer-chromatography (TLC) analysis was performed on silica gel plates (E. Merck silica gel 60 F254 plates) and visualized by ultra-violet (UV) radiation at 254 nm. Flash chromatography for the purification of compound was performed with silica gel as a stationary phase (Merck 60, 230-400 mesh). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses were performed on a Bruker Spectrospin 400 Hz spectrometer. LC-MS analyses were performed on a Waters Alliance 2695 system, eluting in gradient with a flow rate of 0.5 mL/min according to the condition reported

herein in **Table S1**: The analyses were performed on a Monolithic C18 50 X 4.60 mm column by Phenomenex. UV detection was performed on a Diode Array Detector. Mass spectra were registered in both ESI+ and ESI- mode.

Synthesis of novel pyridyl nitrofuranyl isoxazolines with piperazine spacer, 8a-e

Synthesis of tert-butyl 4-(4-vinylphenyl)piperazine-1-carboxylate (3)

A mixture of **1** (1.06 g, 5.80 mmol, 1 eq.), NaO^tBu (2.4 eq.), $PdCl_2[P(o\text{-}Tol)_3]_2$ (0.06 eq.) and **2** (2 eq.) dissolved in toluene (50 mL) was left at reflux at 100°C with stirring in N_2 atmosphere for 3 hours. The crude of reaction was filtered on Celite eluting with DCM and then concentrated under reduced pressure. Purification by column chromatography on silica gel (mobile phase: 9/1, v/v, hexane/EtOAc) provided **3** (1.19 g, 73%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.35 (d, *J* = 8.56 Hz, 2H), 6.89 (d, *J* = 8.56 Hz, 2H), 6.67 (dd, *J* = 10.83, 17.63 Hz, 1H), 5.63 (d, *J* = 17.63 Hz, 1H), 5.13 (d, *J* = 10.83 Hz, 1H), 3.52 - 3.65 (m, 4H), 3.09 - 3.21 (m, 4H), 1.53 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.43, 31.44, 36.51, 49.14, 79.95, 111.22, 116.27, 127.13, 129.66, 136.23, 150.80, 154.72, 162.55. m/z (+EI) calc. for $C_{17}H_{24}N_2O_2$ $(M)^+$ 288.30 found 289.10 $([M]+H)^+$.

Synthesis of N-hydroxy-5-nitrofuran-2-carbimidoyl chloride (5)

5-nitro-2-furaldoxime **4** (1.5 g, 10 mmol, 1 eq.) was dissolved in a solution of HCl 0.5M (1.1 eq., 916 µl) in DMF (10.55 mL) and subsequently oxone (1.1 eq.) was added. The reaction mixture was left under magnetic stirrer at room temperature until TLC showed total consumption of the starting material. The reaction mixture was diluted with water (100 mL) and extracted with

EtOAc (3 x 40 mL). The organic layer was then washed with HCl 0.5 M aqueous solution (100 mL) and brine (100 mL). The collected organic phase was dried on $MgSO_4$, filtered and concentrated under reduced pressure providing **5** (1.87 g, yield was assumed 100%) as a yellow solid.

Synthesis of tert-butyl 4-(4-(3-(5-nitrofuran-2-yl)-4,5-dihydroisoxazol-5-yl)phenyl)piperazine-1-carboxylate (6)

Triethylamine (1.20 eq) was added to a solution of **3** (500 mg, 1.70 mmol 1 eq.) and **5** (1.20 eq.) in $CHCl_3$ (5 mL). The reaction mixture was left stirring in N_2 atmosphere for 20 hours until TLC showed total consumption of the starting material. At that point $CHCl_3$ (11 mL) was added to the reaction mixture and the organic phase was washed with brine (4 x 10 mL) and then dried on $MgSO_4$, filtered and concentrated under reduced pressure. The obtained crude product was purified by column chromatography on silica gel (mobile phase: 7:3, v/v, diethyl ether-hexane) provided **6** (558.60 mg 74.3%) as a deep red solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.29 (d, J = 3.78 Hz, 1H), 7.16 (d, J = 8.56 Hz, 2H), 6.90 (d, J = 4.03 Hz, 1H), 6.82 (d, J = 8.56 Hz, 2H), 5.64 (dd, J = 8.94 Hz, 10.95 Hz, 1H), 3.64 (dd, J = 11.08 Hz, 17.12 Hz, 1H), 3.41 - 3.53 (m, 4H), 3.27 (dd, J = 8.81 Hz, 17.12 Hz, 1H), 2.94 - 3.10 (m, 4H), 1.39 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.43, 41.23, 49.01, 80.02, 83.99, 112.48, 113.15, 116.48, 127.21, 130.17, 147.50, 147.85, 151.60, 154.70. m/z (+EI) calc. for $C_{22}H_{26}N_4O_6$ $(M)^+$ 442.4 found 443.1 $([M]+H)^+$.

2.2 General procedure for synthesis of final products (8a-e)

Concentrated hydrochloric acid solution (14 mL) was added to a solution of **6** (430 mg, 0.97 mmol, 1 eq.) in MeOH (15 mL) and the reaction mixture was left under magnetic stirrer at room temperature for 15 minutes until TLC showed total removal of the protecting group. The solution was then concentrated under reduced pressure to provide **7** (348 mg, 100%) as white solid. Finally, the corresponding bromomethyl pyridine hydrobromide or chlorometyl pyridine hydrochloride derivatives or bromomethyl 4-substitited pyridine derivatives (1.5 eq.) was added to a solution of **7** (from 61 mg to 177 mg, 1 eq.) in DMF (8 mL) in the presence of K_2CO_3 (3 eq.). The reaction mixture was stirred at 50°C under N_2 atmosphere for 16 hours. The reaction was quenched by addition of water (20 mL) and subsequently extracted with EtOAc (3 x 20 mL). The collected organic phases were washed with brine (3X15 mL), dried on $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (mobile phase: from 98:2, v/v, DCM-MeOH to 95:5, v/v, DCM-MeOH) obtaining the final products **8a-e**.

3-(5-nitrofuran-2-yl)-5-(4-(4-(pyridin-2-ylmethyl)piperazin-1-yl)phenyl)-4,5-dihydroisoxazole (8a).

Obtained 68 mg (98%), as a dark yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.57 (d, J = 4.78 Hz, 1H), 7.59 - 7.71 (m, 1H), 7.43 (d, J = 7.81 Hz, 1H), 7.37 (d, J = 3.78 Hz, 1H), 7.12 - 7.25 (m, 3H), 7.00 (d, J = 4.03 Hz, 1H), 6.90 (d, J = 8.81 Hz, 2H), 5.72 (dd, J = 8.94 Hz, 10.95 Hz, 1H), 3.64 - 3.79 (m, 3H), 3.37 (dd, J = 8.94 Hz, 17.25 Hz, 1H), 3.14 - 3.28 (m, 4H), 2.58 - 2.71 (m, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 41.15, 48.67, 53.14, 64.55, 84.12, 112.44, 113.15, 115.91, 122.23, 123.40, 127.16, 129.44, 136.51, 147.56, 147.85, 149.40, 151.68. HRMS (EI, m/z): calc. for $C_{23}H_{24}N_5O_4$ $([M]+H)^+$ 434.1823 found 434.1819.

3-(5-nitrofuran-2-yl)-5-(4-(4-(pyridin-3-ylmethyl)piperazin-1-yl)phenyl)-4,5-dihydroisoxazole (8b).

Obtained 44 mg (33.3%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.51 (s, 1H), 8.46 (d, J = 4.78 Hz, 1H), 7.65 (d, J = 7.81 Hz, 1H), 7.31 (d, J = 3.78 Hz, 1H), 7.12 - 7.25 (m, 3H), 6.94 (d, J = 3.78 Hz, 1H), 6.83 (d, J = 8.56 Hz, 2H), 5.66 (dd, J = 8.94 Hz, 10.95 Hz, 1H), 3.66 (dd, J = 11.08, 17.12 Hz, 1H), 3.50 (s, 2H), 3.30 (dd, J = 9.06 Hz, 17.12 Hz, 1H), 3.04 - 3.20 (m, 4H), 2.42 - 2.59 (m, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 41.17, 48.71, 52.90, 60.17, 84.09, 112.45, 113.16, 115.95, 123.42, 127.18, 129.56, 133.32, 136.79, 147.54, 147.85, 148.81, 150.51, 151.60. HRMS (EI, m/z): calc. for $C_{23}H_{24}N_5O_4$ $([M]+H)^+$ 434.1823 found 434.1863.

3-(5-nitrofuran-2-yl)-5-(4-(4-(pyridin-4-ylmethyl)piperazin-1-yl)phenyl)-4,5-dihydroisoxazole (8c).

Obtained 104 mg (51%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.51 - 8.61 (m, 2H), 7.39 (d, J = 3.78 Hz, 1H), 7.31 (d, J = 5.54 Hz, 2H), 7.23 - 7.27 (m, J = 8.81 Hz, 2H), 7.02 (d, J = 3.78 Hz, 1H), 6.86 - 6.95 (m, J = 8.56 Hz, 2H), 5.75 (dd, J = 9.06 Hz, 11.08 Hz, 1H), 3.74 (dd,

J = 11.20 Hz, 17.25 Hz, 1H), 3.57 (s, 2H), 3.39 (dd, *J* = 8.94 Hz, 17.25 Hz, 1H), 3.19 - 3.28 (m, 4H), 2.57 - 2.67 (m, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 41.15, 48.72, 53.04, 61.67, 84.08, 112.52, 113.16, 115.91, 123.88, 127.19, 129.58, 147.34, 147.51, 147.83, 149.87, 151.58, 152.10. HRMS (EI, m/z): calc. for $C_{23}H_{24}N_5O_4$ $([M]+H)^+$ 434.1823 found 434.1820.

5-(4-(4-((6-fluoropyridin-3-yl)methyl)piperazin-1-yl)phenyl)-3-(5-nitrofuran-2-yl)-4,5-dihydroisoxazole (8d).

Obtained 11 mg (10.6%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.16 (d, *J* = 1.89 Hz, 1H), 7.83 (td, *J* = 7.96 Hz, 1.95 Hz, 1H), 7.40 (d, *J* = 3.78 Hz, 1H), 7.25 (d, *J* = 8.69 Hz, 2H), 7.03 (d, *J* = 3.90, 1H), 6.87 – 6.97 (m, 3H), 5.75 (dd, *J* = 11.02 Hz, 9.00 Hz, 1H), 3.75 (dd, *J* = 17.12 Hz, 11.08 Hz, 1H), 3.57 (s, 2 H), 3.39 (dd, *J* = 17.18 Hz, 8.88 Hz, 1H), 3.18 – 3.26 (m, 4H), 2.56 – 2.66 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 41.13, 48.62, 52.76, 59.06, 84.02, 109.15, 109.53, 112.44, 113.13, 115.94, 127.14, 129.63, 141.99, 142.07, 147.47, 147.73, 147.81, 147.86, 151.47, 161.91.

5-(4-(4-((6-methoxypyridin-3-yl)methyl)piperazin-1-yl)phenyl)-3-(5-nitrofuran-2-yl)-4,5-dihydroisoxazole (8e)

Obtained 37 mg (34.6%) as a yellow solid. Obtained 37 mg (34.6%) as a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.08 (d, *J* =2.14 Hz, 1H), 7.58-7.67 (m, 1H), 7.40 (d, *J* = 3.90 Hz, 1H), 7.25 (d, *J* = 8.69 Hz, 2H), 7.04 (d, *J* = 3.78 Hz, 1H), 6.91 (d, *J* = 8.81 Hz, 2H), 6.74 (d, *J* = 8.44 Hz, 1H), 5.75 (dd, *J* = 11.02 Hz, 9.00 Hz, 1H), 3.94 (s, 3H), 3.75 (dd, *J* = 17.25 Hz,

11.08 Hz, 1H), 3.51 (br. s., 2H), 3.40 (dd, *J* = 17.25 Hz, 8.94 Hz, 1H), 3.22 (br. s., 4H), 2.60 (br. s., 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 41.11, 48.60, 52.65, 53.41, 59.47, 84.04, 110.69, 112.41, 113.11, 115.87, 125.65, 127.11, 129.45, 139.87, 147.08, 147.48, 147.80, 151.55, 152.03, 163.64.

Synthesis of novel pyridyl nitrofuranyl isoxazolines without the piperazine spacer, 10a-c

Synthesis of 5-(4-bromophenyl)-3-(5-nitrofuran-2-yl)-4,5-dihydroisoxazole (9).

To a solution of 4-bromostyrene (400 mg, 2.18 mmol, 1 eq.) in $CHCl_3$ (10 mL), 0.36 mL (1.20 eq) trimethylamine and **5** (500 mg, 2.62 mmol, 1.20 eq) were added. The reaction mixture was stirred overnight under N_2 atmosphere. The organic phase was extracted with $CHCl_3$ (3 x 10 mL), washed with brine and dried over $MgSO_4$, filtered and concentrated under reduced pressure. The obtained crude product was purified by column chromatography on silica gel (mobile phase: 1:1, v/v, diethyl ether-hexane) provided **9** (515 mg 70%) as a cream coloured solid.

^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 7.52 (d, J = 8.31 Hz, 2H), 7.39 (d, J = 3.78 Hz, 1H), 7.24 (d, J = 8.56 Hz, 2H), 7.04 (d, J = 4.03 Hz, 1H), 5.79 (dd, J = 8.18 Hz, 11.20 Hz, 1H), 3.84 (dd, J = 11.33 Hz, 17.12 Hz, 1H), 3.35 (dd, J = 8.18 Hz, 17.25 Hz, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 147.58, 146.85, 138.44, 131.98, 127.40, 122.59, 112.98, 112.81, 82.91, 41.70, 29.61. m/z (+EI) calc. for $C_{13}H_9BrN_2O_4$ $(M)^+$ 335.9 found 336.9 $([M]+H)^+$.

Synthesis of 3-(5-nitrofuran-2-yl)-5-(4-(pyridin-2-yl)phenyl)-4,5-dihydroisoxazole (10a).

Tetrakis(triphenylphosphine)palladium(0) (69.2 mg, 0.06 mmol, 0.08 eq.), K_2CO_3 (290.20 mg, 2.10 mmol, 3 eq.) and pyridine-2-boronic acid (129.04 mg, 1.05 mmol, 1.50 eq.) were added to a

solution of **9** (262.95 mg, 0.70 mmol, 1 eq.) in dioxane (5 mL) and water (0.50 mL). The reaction mixture was stirred at reflux under an N_2 atmosphere for 72 hrs. The reaction was not completed, but it was decided to work up the reaction mixture to prevent formation of further impurities. The reaction was filtered on a Celite pad, with the solvent evaporating under reduced pressure and the crude reaction mixture dissolving in ethyl acetate (10 mL). This solution was washed with water and the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The obtained crude product was then purified by column chromatography on silica gel (mobile phase: from 100% DCM to 95/5, v/v, DCM/acetone), obtaining the final product **10a** as a yellow-red solid.

1H NMR (400 MHz, $CDCl_3$) δ: 8.74 (d, J = 5.54 Hz, 1H), 8.04 (d, J = 8.31 Hz, 2H), 7.73 - 7.83 (m, 2H), 7.49 (d, J = 8.31 Hz, 2H), 7.41 (d, J = 3.78 Hz, 1H), 7.30 (d, J = 6.04 Hz, 1H), 7.07 (d, J = 3.78 Hz, 1H), 5.91 (dd, J = 8.31 Hz, 11.33 Hz, 1H), 3.88 (dd, J = 11.08 Hz, 17.12 Hz, 1H), 3.45 (dd, J = 8.56 Hz, 17.12 Hz, 1H). HRMS (EI, m/z): calc. for $C_{18}H_{13}N_3O_4$ $(M)^+$ 336.0979 found 336.0979($[M]+H)^+$.

2.3 General procedure for the synthesis of pyridyl isoxazolines without the piperazine linker (10b-c)

$[Pd_2(dba)_3]$ (48.9 mg, 0.053 mmol, 0.067 eq.), Tricyclohexylphosphine (131.24 mg, 0.47 mmol, 0.6 eq.), K_3PO_4 (496.71 mg, 2.34 mmol, 3 eq.) and the desired pyridine boronic acid (143.80 mg, 1.17 mmol, 1.5 eq.) were added to a solution of **9** (262.95 mg, 0.70 mmol, 1 eq.) in dioxane (5 mL) and water (0.50 mL). The reaction mixture was stirred at reflux under N_2 atmosphere for 20h at which point LC-MS monitoring showed complete consumption of the starting material. The reaction was then filtered on a Celite pad, the solvent evaporated under reduced pressure and the evaporated crude reaction mixture was dissolved in ethyl acetate (10 mL). This solution was

washed with water and the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The obtained crude product was then purified by column chromatography on silica gel (mobile phase: from 100% DCM to 95/5, v/v, DCM/acetone) obtaining the final products **10b-c**.

3-(5-nitrofuran-2-yl)-5-(4-(pyridin-3-yl)phenyl)-4,5-dihydroisoxazole (10b).

Obtained 24 mg as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.85 (d, J = 2.27 Hz, 1H), 8.62 (dd, J = 1.51 Hz, 4.78 Hz, 1H), 7.89 (td, J = 1.92 Hz, 7.99 Hz, 1H), 7.62 (d, J = 8.31 Hz, 2H), 7.49 (d, J = 8.31 Hz, 2H), 7.35 - 7.44 (m, 2H), 7.07 (d, J = 4.03 Hz, 1H), 5.90 (dd, J = 8.31 Hz, 11.33 Hz, 1H), 3.88 (dd, J = 11.20 Hz, 17.25 Hz, 1H), 3.45 (dd, J = 8.31 Hz, 17.12 Hz, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 148.97, 148.42, 148.04, 147.41, 139.73, 138.57, 136.24, 134.78, 128.02, 126.89, 123.98, 113.34, 113.05, 83.66, 42.14, 30.00. HRMS (EI, m/z): calc. for $C_{18}H_{13}N_3O_4$ $(M)^+$ 336.0979 found 336.0976 $([M]+H)^+$.

3-(5-nitrofuran-2-yl)-5-(4-(pyridin-4-yl)phenyl)-4,5-dihydroisoxazole (10c).

Obtained 17.40 mg as a yellow glassy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.68 (d, J = 5.54 Hz, 2H), 7.68 (d, J = 8.31 Hz, 2H), 7.46 - 7.55 (m, 4H), 7.41 (d, J = 3.78 Hz, 1H), 7.08 (d, J = 3.78 Hz, 1H), 5.91 (dd, J = 8.18 Hz, 11.20 Hz, 1H), 3.89 (dd, J = 11.33 Hz, 17.12 Hz, 1H), 3.45 (dd, J = 8.06 Hz, 17.12 Hz, 1H). HRMS (EI, m/z): calc. for $C_{18}H_{13}N_3O_4$ $(M)^+$ 335.3 found 336.0977 $([M]+H)^+$. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 150.44, 148.03, 147.35, 140.79, 138.83, 127.93, 126.89, 121.95, 113.34, 113.08, 83.54, 42.19, 30.00. HRMS (EI, m/z): calc. for $C_{18}H_{13}N_3O_4$ $(M)^+$ 336.0979 found 336.0977 $([M]+H)^+$.

2.3 Toxicity evaluation of the compounds against eukaryotic cells

2.3.1 Cell Culture

HeLa (human cervical cancer) cell line and non-tumour WI38 cell line was obtained from the American Type Culture Collection. The HeLa cell-line was maintained in monolayer culture in 75 cm^2 flasks (TPP, Switzerland) under a humidified 5% CO_2 atmosphere at 37°C. The HeLa cell line was maintained in Dulbecco's Modified Eagles Media (DMEM; Invitrogen) supplemented with foetal bovine serum (10% v/v; Invitrogen), L-glutamine (2 mM; Invitrogen), non-essential amino acids (1x; Invitrogen) and Penicillin-Streptomycin (1% v/v, Invitrogen). For WI 38 cell line, Dulbecco's MEM, supplemented with L-glutamine (2mM; Invitrogen), non-essential amino acids (1x; Invitrogen), penicillin-streptomycin (1% v/v, Invitrogen) and foetal bovine serum (15%, Biosera UK) was used. For seeding, cells were counted using a Neubauer haemocytometer (Assistant, Germany) by microscopy (Nikon, USA) on a non-adherent suspension of cells that were washed in PBS, trypsinised, centrifuged at 4°C at 4000 rpm for 5 min and re-suspended in fresh medium.

2.3.2 MTT Assay

The cells were grown in normal cell culture conditions at 37 ºC under a 5% CO_2 humidified atmosphere using appropriate medium. The cell count was adjusted to 10^5 cells/mL and 10,000 cells were added per well. The cells were incubated for 24 hours and 1 µl of the appropriate ligand concentrations were added to the wells in triplicates. After 96 hours of continuous exposure to each compound, the cytotoxicity was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Lancaster Synthesis Ltd, UK) colorimetric

assay.[34 Absorbance was quantified by spectrophotometry at λ = 570 nm (Envision Plate Reader, PerkinElmer, USA). IC_{50} values were calculated by a dose-response analysis using the Prism GraphPad Prism® software.

2.4 Microbiological Evaluation of the Compounds

2.4.1 Determination of minimum inhibitory concentration

MICs were determined using the broth microdilution method as outlined in the CLSI guidelines [22]. Cell growth in Muller Hinton Broth (MHB) was determined by measuring optical density (600 nm) using a FLUOstar Omega microplate reader (BMG Labtech). The MIC was determined as the lowest concentration of drug at which growth was below an optical density of 0.10 at 600 nm after 20 hours growth. Tests were conducted in triplicate.

2.4.2 Determination of minimum bactericidal concentration

To determine the minimum bactericidal concentration (MBC), 10ul was taken from all wells of the MIC plate at and above the MIC and spotted onto a tryptic soy agar plate. The agar plates were incubated at 37 °C for 24 hours and the MBC was defined as the concentration at which no colonies were seen. The ratio MBC/MIC that was used to evaluate if the compound is bactericidal (MBC/MIC = 1 or 2) or bacteriostatic (MBC/MIC = 4 or 16).

2.4.3 Time kill assays.

Flasks of MHB were inoculated with test organism at a concentration ~10^6 cfu/mL in a total volume of 50 mL. The antimicrobial agents were then added at a concentration of 4 x MIC and incubated at 37 °C in a shaking incubator at 200 rpm. Samples (0.10 mL) were taken from each

sample every hour for 8 hours and at 24 hours following inoculation. The effect on bacterial growth was determined using a modification of the Miles Misra dilution methods to estimate viable counts.

3.0 Results

3.1 Chemistry

The synthetic scheme for the pyridyl nitrofuranyl isoxazoline compounds with piperazine spacer (**8a-e**) is shown in **Fig 2**. First, the piperazine spacer was installed on p-bromo styrene by a palladium-catalyzed aromatic amination reaction[23] on **1** with N-Boc piperazine in 73% yield. Next, the 5-nitro-2-furaldoxime **4** was converted to the corresponding hydroximoyl chloride **5** using Oxone and HCl[24] in DMF which upon treatment with olefin 3, underwent a [3 + 2] regioselective cycloaddition[25] under inert condition to give the reference Boc-protected isoxazoline **6** in 74.3% yield. The Boc-deprotection of **6** was carried out in a methanolic solution of HCl in room temperature in quantitative yield. Finally, the free amine **7** was treated with 2- and 3-bromomethyl pyridine, 4-chloromethyl pyridine, 5-(bromomethyl)-2-fluoropyridine, 5-(bromomethyl)-2-methoxypyridine, in the presence of K_2CO_3 to afford **8a**, **8b 8c, 8d and 8e** with between 98%, 51%, 33%, 10.6% and 34.6% yield, respectively. Compounds **8a-c** were initially synthesized and evaluated for their antimicrobial activity. **8d and 8e** are structural analogues of **8b** with an electron withdrawing F and electron donating methoxy group at 2-position of the pyridine ring. The aim of this modification was to explore the role of electronic environment in the terminal pyridyl group on the antimicrobial activity of this class of compounds.

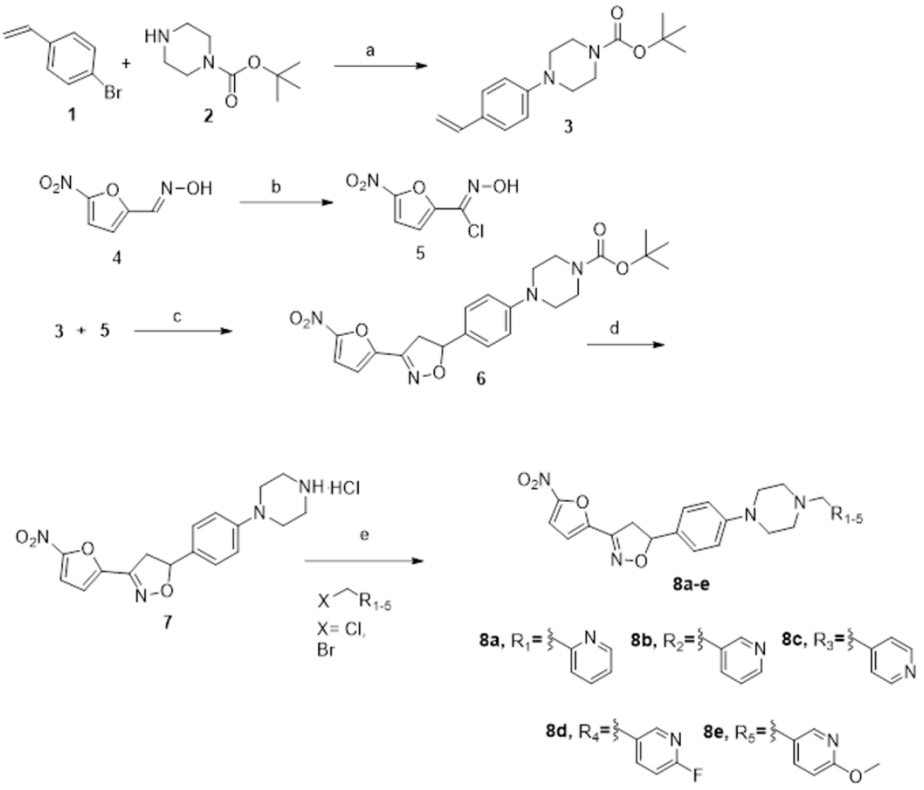

Fig 2. Synthesis of pyridyl nitrofuranyl isoxazolines with piperazine spacer 8a-e. Reagent and conditions: a) $PdCl_2[P(o\text{-}Tol)_3]_2$, NaO*t*Bu, toluene, reflux, 3 hrs; b) Oxone, HCl 0.5M aqueous solution, DMF, r.t., 24 hrs; c) Et_3N, chloroform, r.t., 20 hrs; d) HCl 37% aqueous solution, MeOH, r.t., 2 hrs; e) K_2CO_3, DMF, 50 ^oC, overnight.

To explore the role of the piperazine spacer between the phenyl ring and the terminal pyridyl ring, three more compounds were designed in which the piperazine spacer was removed and the phenyl ring was directly coupled to the pyridyl ring using Suzuki coupling. These compounds (**10a-c**) were synthesized according to synthetic scheme reported in **Fig 3**. First, the 5-nitro-2-

furaldoxime **4** was converted to the corresponding hydroximoyl chloride **5** using Oxone and HCl[24] in DMF which underwent a [3 + 2] regioselective cycloaddition[25] with p-bromo styrene under inert condition to afford the Suzuki substrate **9** in 70% yield. Finally, the Suzuki cross-coupling reactions between **9** and the pyridyl-3-boronic acid and pyridyl-4-boronic acid were carried out using $Pd_2(dba)_3$ as a catalyst under inert condition in the presence of tricyclohexylphosphine and K_3PO_4 to afford **10b**, and **10c**. The Suzuki reaction between **9** and pyridyl-2-boronic acid did not proceed under same condition, and was carried out using tetrakis palladium as the catalyst with a modest 10% yield to afford **10a**.

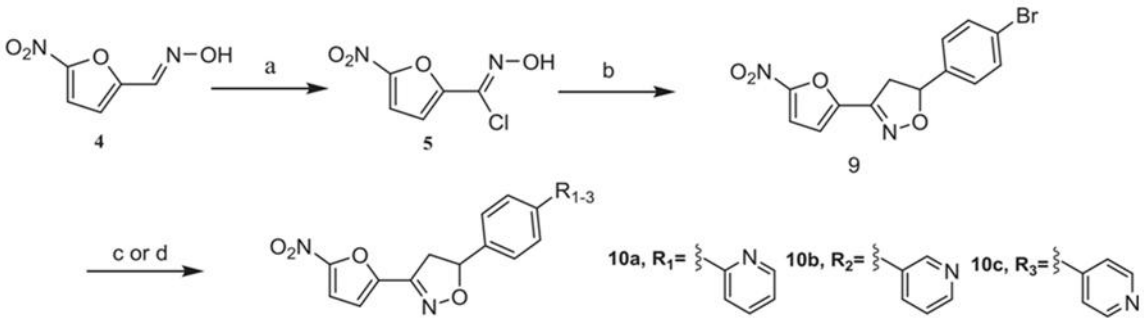

Fig 3. Synthesis of pyridyl nitrofuranyl isoxazolines without the piperazine spacer 10a-c. Reagent and conditions: a) Oxone, HCl 0.5M aqueous solution, DMF, r.t., 24 hrs; b) bromostyrene, Et_3N, chloroform, r.t., 20 hrs; c) $[Pd_2(dba)_3]$, K_3PO_4, $P(Cy)_3$, Dioxane, H_2O 100 °C, d) $Pd(PPh_3)_4$, K_2CO_3, Dioxane/H_2O, 100 °C

3.2 Microbiological Evaluation

3.2.1 MIC and MBC Determination

The antibacterial evaluation of compound **6, 8a—c** and **10a-c** were carried out using the broth microdilution method as outlined in the CLSI guidelines[22]. Initially, the compounds were tested against a panel of ESKAPEE pathogens consisting of three Gram-positive *Staphylococcus* and six Gram-negative bacteria with a variety of antibiotic resistance mechanisms. The reference isoxazoline **6**, was found to be broadly inactive against both Gram-positive and Gram-negative panel while newly synthesized pyridyl nitrofuranyl isoxazolines with piperazine spacer **8a-b** showed a wide range of activity against the Gram-positive panel with MICs between 16 to >128 µg/mL (**Table 1**), and **8c** showed activity similar to that of **6 (Table 1)**. None of the compounds showed any activity against the Gram-negative pathogens tested (*Klebsiella pneumoniae* strains NCTC 13368 and M6[26], *Acinetobacter baumannii* strains ATCC BAA-1710 and ATCC 17978, *Pseudomonas aeruginosa* strains PAO1 and NCTC 13437; MIC values all >128 µg/mL; results not shown). The initial Gram-positive panel included; methicillin sensitive (MSSA) *S. aureus* MSSA4144, and methicillin resistant *S. aureus* (MRSA) isolates representative of EMRSA-15 and EMRSA-16[27].

Table 1. Activity of 8a-e against the initial *Staphylococcus* panel.

	MIC (µg/ml)									
Bacteria	**6**	**8a**	**8b**	**8c**	**8d**	**8e**	**Nitrofur - antoin**	**Cipro**	**Vancomyc in**	**Amoxicil lin**
MSSA 9144	32	16	16	16	32	32	16	0.125	0.5	0.1-2
EMRSA -15	>128	32	16	>128	128	128	16	32	2	128
EMRSA -16	>128	32	32	128	16	32	16	32	2	>128

Compound **8b** gave MIC values of 16-64 μg/mL for MSSA/MRSA strains. **8b** showed comparable activity against the MSSA 9144 and EMRSA 15 strains compared to the nitrofuran group containing control antibiotic nitrofurantoin (MIC 16 μg/mL) which is expected to share a similar mechanism of action (i.e., nitrocompound toxicity). This prompted us to synthesize two additional analogues of 8b with weakly electron withdrawing F containing **8d** and electron donating methoxy group containing **8e**. **8d** showed an improvement in MIC value against EMRSA-16 (MIC 16 μg/mL) compared to 8b, comparable activity against MSSA9144 but notably lost activity against EMRSA-15. Compound **8e** also showed a similar profile except an improvement against EMRSA-16 wasn't observed for this analogue. The MIC values of the shortened pyridyl nitrofuranyl isoxazolines without the piperazine spacer are reported in **Table 2.** Compound **10a** was found to be inactive against the *Staphylococcus* panel. Compound **10b** showed activity against all three *Staphylococcus* strains with MIC values between 16 to 128 μg/mL. Compound **10c** also showed activity against all three *Staphylococcus* strains but the MIC values were slightly higher (MIC range 64 to 128 μg/mL) than **10b**. It appeared that the removal of the piperazine spacer had a detrimental effect on the MIC values of **8a** and **8b**, but had a small positive effect on the MIC values of **8c**. Compound **10b** maintained comparable activity against methicillin resistant *Staphylococcus* EMRSA 16 compared to nitrofurantoin.

Table 2. Activity of 10a-c against the initial *Staphylococcus* panel.

	MIC (µg/ml)						
Bacteria	**10a**	**10b**	**10c**	**Nitrofurantoin**	**Cipro**	**Vancomycin**	**Amoxicillin**
MSSA 9144	>128	32	64	16	0.125	0.5	0.1-2
EMRSA - 15	>128	64	128	16	32	2	128
EMRSA-16	>128	16	64	16	32	2	>128

Compounds **8a** and **8b** were tested for their minimum bactericidal concentration (MBC) and the ratio of MBC to MIC was calculated to determine the bactericidal vs bacteriostatic mode of action the compounds. Both **8a** and **8b** showed MBC of ≥128 µg/mL against the MSSA 9144 strain with an MBC to MIC ratio of 8 suggesting a bacteriostatic mode of action as compounds are usually regarded bactericidal if the MBC is no more than four times MIC. Both compounds showed MBC values of >128 µg/mL against the EMRSA strains with MBC to MIC ratio of greater than 4 in each cases.

Compounds **8a** and **8b** were tested against an extended panel of *S. aureus* and *S. epidermidis* strains from a range of different lineages (**Table 3**). Both **8a** and **8b** showed good activity against the extended *Staphylococcus* panel with MICs ranging from 8 to 32 µg/mL for **8a** and 4-16 µg/mL for **8b**. Both compound **8a** and **8b** showed either improved or comparable activities (MICs 4-16 µg/mL) against the extended *Staphylococcus* panel compared to nitrofurantoin (MICs 16 µg/mL). The levels of activity against different strains were unaffected by molecular mechanisms which mediate resistance to ciprofloxacin and amoxicillin in Staphylococcus spp (e.g. strains EMRSA-15 and EMRSA-16).

Table 3. **Activity of 8a and 8b against extended *Enterococcus* and *Staphylococcus* panels.**

		MIC (µg/ml)					
	Bacteria	**8a**	**8b**	**Nitrofurantoin**	**Cipro**	**Vancomycin**	**Amoxicillin**
Extended Staphylococcus panel	***S. epidermidis* ATCC 35984**	8-16	4-8	16	0.125-0.5	4	128
	***S. aureus* A1988**	16	8	16	0.25	2	>128
	***S. aureus* BGW541**	8-16	8	16	0.5	2	0.25-0.5
	MRSA NCTC 12493	8	4-8	16	0.5	1	>128
	***S. aureus* 13142**	8-16	8-16	16	0.25	2	0.25
	***S. aureus* 6571 PVL**	16-32	16	16	0.25-0.5	2	0.25
	***S. aureus* 8325 PVL**	16	16	16	0.125-0.25	2	0.25-0.5

3.2.2 Time Kill Assay

Compound **8b** was selected for time kill analysis against the EMRSA-15 strain to determine whether **8b** was bactericidal or bacteriostatic. **8b** appeared to be bacteriostatic against with activity against EMRSA-15 comparable to that of ciprofloxacin which was used as a control (**Fig4**). There was no evidence of resistant colonies being selected for during the time-kill assays, but there were viable cells which survived the treatment process with **8b** for EMRSA-15 with no significant reduction in the viable count between approximately 5 hours post treatment and the end of the time course at 24 hours (**Fig 4**). The data suggests a bacteriostatic mode of action which is consistent with the MBC to MIC ratio reported in Table 3.

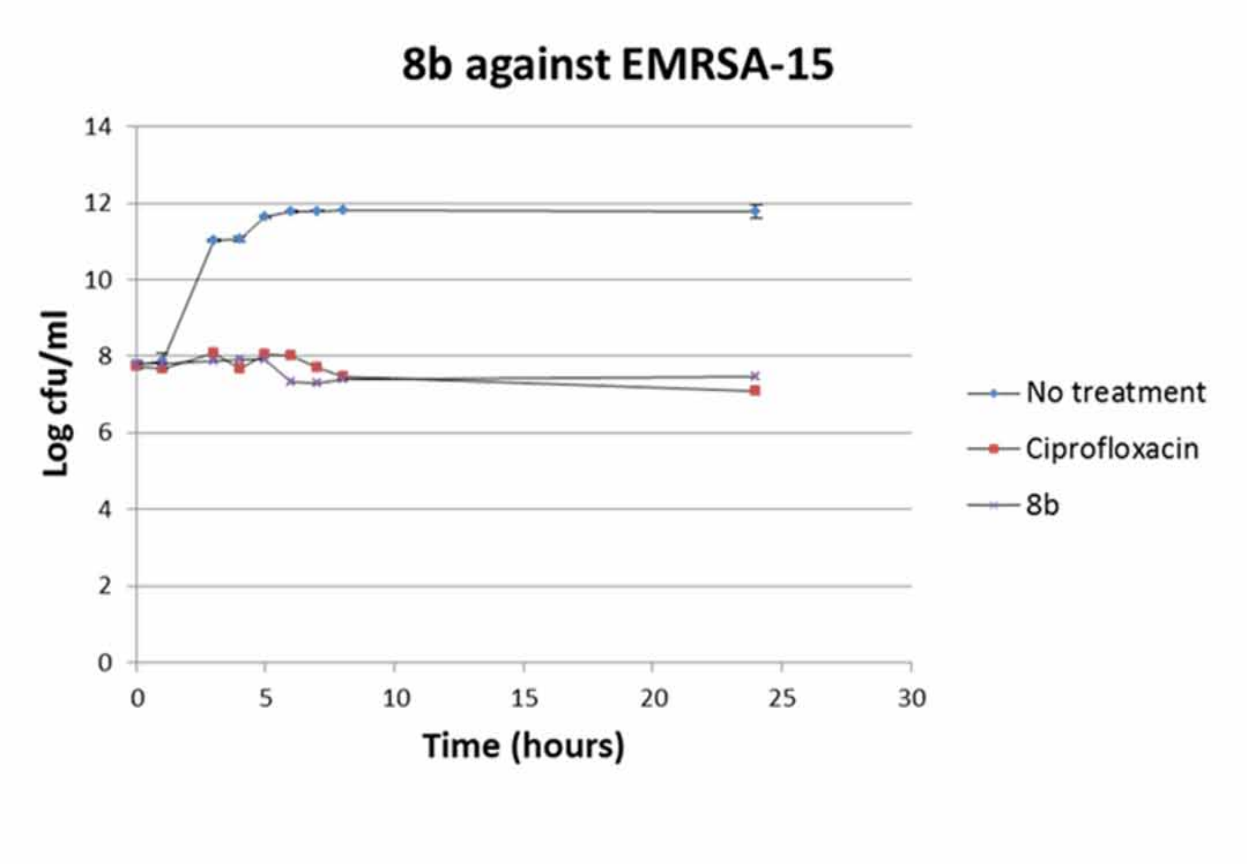

Fig 4. Time kill analysis of compound 8b against *Staphylococcus* strain EMRSA-15. EMRSA-15 was treated with **8b** or ciprofloxacin at 4X MIC for 24 hours at 37 °C. Results are the average of at least three replicate experiments and error bars show the standard error of the mean.

3.2.3 Cytotoxicity Screening

Finally, the compounds were tested for their cytotoxic potential against the cervical cancer cell line HeLa using the MTT assay[28] and the non-tumour lung fibroblast cell line WI-38 and the results are shown in **Fig 5** and S1Table. Doxorubicin was used as a positive control in the MTT assay for comparison purpose. Doxorubicin showed notable cytotoxicity with an IC_{50} of 71 nM (**Fig 5**), but none of the compounds demonstrated a measurable IC_{50} at the concentrations tested

and this lack of toxicity against the mammalian cells was encouraging from the anti-infective point of view as this chemical scaffold offers selective toxicity against prokaryotic cells.

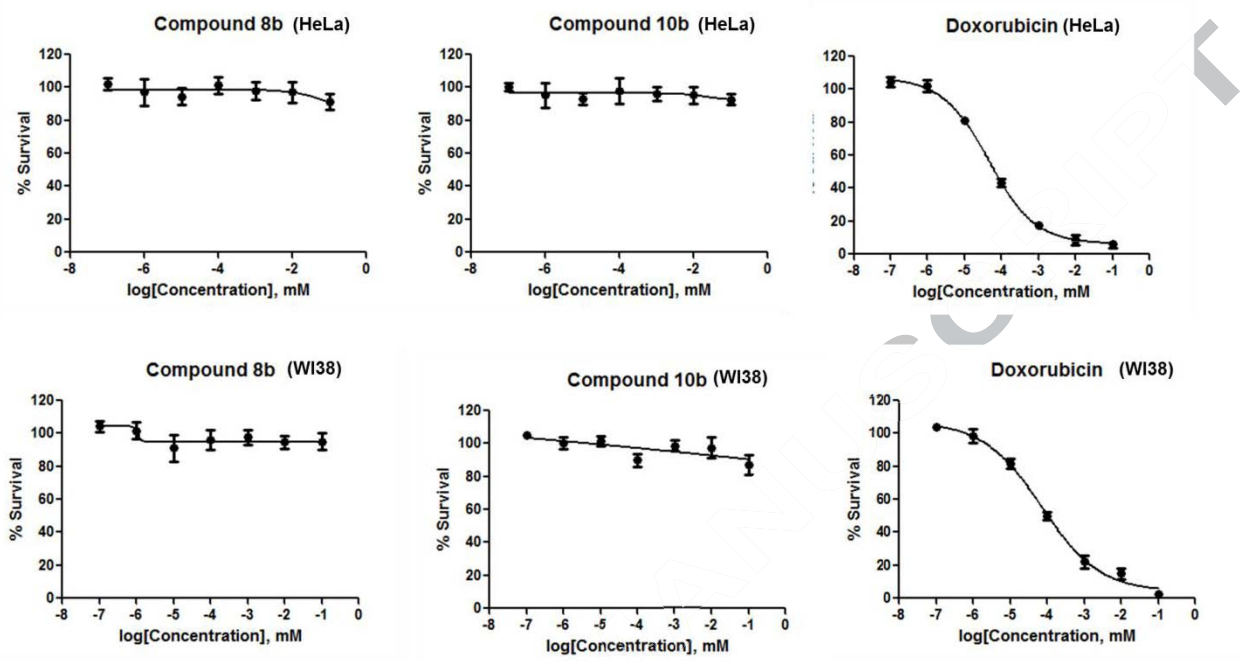

Fig 5. MTT cell-viability assay profile in HeLa and WI-38 cells treated with 8b, 10b and control compound doxorubicin. HeLa and WI-38 cells were treated with selected compounds for 96 hours. Results are the average of at least three replicate experiments.

4.0 Discussion

Eight new pyridyl nitrofuranyl isoxazolines were successfully synthesized using solution phase linear chemistry. The compounds were derived from a reported molecule that has shown antitubercular activity but was found to be broadly inactive against both Gram-positive and Gram-negative bacteria. Compounds generated by modifying the terminal phenyl ring and heterocyclic spacer (**8a-b, d, e**, **10a-c**) showed significant activity against MDR Gram-positive pathogens and no notable toxicity against eukaryotic cell lines tested. Although the presence of the piperazine spacer wasn't crucial for activity of these compounds against Gram-positive

bacteria, compounds with piperazine spacer showed slightly better activity compared to analogues without the piperazine spacer. The position of the N-atom within the terminal pyridine ring played a crucial role in conferring activity to these molecules and 3-pyridyl analogues (**8b** and **10b**) were significantly more potent compared to 2-pyridyl and 4-pyridyl analogues for both sets of compounds (i.e., compounds with and without piperazine spacer). Electron donating and electron withdrawing substituents in the terminal pyridine ring maintained or enhanced activity against MSSA-9144 and EMRSA-16 strain but a loss of activity against EMRSA-15 strain was observed in both cases. This SAR information will be helpful in designing future analogues of this chemical class. The incorporation of the nitrofuran ring suggests that the compounds may be active via nitrocompound toxicity, akin to that mediated by nitrofurantoin and benzothiazinone-based compounds[29].

Compound **8b** showed good activity against a wider panel of multiple drug-resistant Staphylococcus strains. Many of the strains in the extended panel are multidrug resistant and have a range of diverse resistance mechanisms. For example, strain A1988 is resistant to trimethoprim, muciprocin, oxacillin, amoxicillin, gentamicin and kanamycin. No significant difference in sensitivity was observed for strains 6571 and 8325 which express Panton Valentin Leukocidin (PVL), which is a virulence factor associated with aggressive infection in healthy individuals. Compound **8b** showed similar activity (MIC 4-16 μg/mL) against most members of the extended panel.

The increased susceptibility of Gram-positive strains compared to Gram-negative bacteria mirrors other recent investigations of nitrofuran-containing compounds, such as N-

acylhydrazone[30, 31], which have been investigated for the control of nosocomial pathogens. The reduced efficacy against Gram-negative pathogens probably reflects the greater barrier functions of the bacterial membrane, but may also be influenced by cellular metabolism related to nitrocompound activation and/or toxicity. Given the likely mechanisms of action of the pyridyl nitrofuranyl isoxazolines being investigated here, we would not have expected any of the existing resistance mechanisms in the Gram-positive strain panel to mediate cross-resistance to the new compounds. Indeed there was no difference in the MIC levels (defined here as being less than 4-fold difference in MIC) determined for strains resistant or sensitive to amoxicillin, vancomycin or ciprofloxacin, respectively. Hence the compounds are equally active against isolates which are resistant to key front-line antibiotics. The MICs of the pyridyl nitrofuranyl isoxazolines are consistent with those of other novel nitrofuran-containing compounds tested against Gram-positive pathogens[30, 31]. The absolute MIC values for drug resistant strains that were susceptible against **8b** were consistently lower for compound **8b** (typically 4-16 μg/mL) than for the control antibiotic nitrofurantoin which may have a similar mechanism of action (i.e., nitrocompound toxicity) and shares structural similarity. Nitrofurantoin is used routinely in the United Kingdom and elsewhere for the treatment of urinary tract infections and the improved activity of **8b** compared to nitrofurantoin is both encouraging and significant from a drug-discovery point of view as there is significant opportunity to make medicinal chemistry modifications on the scaffold to develop more potent antibacterial agents. It should be noted that the MIC values of **8b** were higher than that for ciprofloxacin (0.125-2 μg/mL), vancomycin (0.5-2 μg/mL) or amoxicillin (0.25-2 μg/mL), Given the different mode of action of these antibiotics, this is unsurprising and does not necessarily preclude the development of pyridyl nitrofuranyl isoxazolines as candidate antibiotics, especially given their low eukaryotic cell toxicity. Like

nitrofurantoin, there are several antibiotics which are used routinely in the clinic and where the MIC for susceptible strains is higher than that observed for compound **8b**. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) define resistance to nitrofurantoin as being an MIC of 64 μg/mL and above, for *Staphylococcus spp*. In contrast, *S.aureus* strains with an MIC of 2 μg/mL are defined as being resistant to ciprofloxacin. There is significant opportunity to make medicinal chemistry modifications on the scaffold, to develop more potent antibacterial agents.

Although this chemical scaffold showed a relatively narrow spectrum of activity, there is increasing support within the clinical community for this since they do not have wide ranging deleterious effects on the patients' normal flora. Narrow spectrum agents can eliminate resistance pressure on large sections of the microbiome, as these agents will be inactive against them, and thereby play a pivotal role in minimising spread or resistance[32]. The number of pipeline drugs presently undergoing clinical trials against MRSA is not particularly encouraging[33] and, although the pipeline is not completely empty, it is important to exploit existing as well as new chemical scaffolds to develop drug leads that can be optimized into clinical candidates.

5.0 Conclusion

Concern over antibiotic resistance is growing, and novel classes of antibiotics are needed. We have successfully modified a chemical scaffold that has previously shown activity against *Mycobacterium* species, but was inactive against MDR Gram-positive pathogens, and introduced notable activity against MRSA strains. It was possible to obtain limited SAR information from

the compound set and this information can be useful to generate future compounds of this type. The pyridyl nitrofuranyl isoxazoline **8b** showed improved activity against a wider panel of drug-resistant and sensitive *Staphylococcus* strains compared to nitrofurantoin which like these compounds shares nitrofuran group and may have a similar mechanism of action. The lack of mammalian toxicity and the opportunity to make medicinal-chemistry modifications make pyridyl nitrofuranyl isoxazoline scaffold an interesting starting point to develop more potent antibacterial agents against MDR Gram-positive pathogens.

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References

[1] C.A. Arias, B.E. Murray, Antibiotic-Resistant Bugs in the 21st Century -- A Clinical Super-Challenge, New England Journal of Medicine, 360 (2009) 439-443.

[2] M.A. Fischbach, C.T. Walsh, Antibiotics for Emerging Pathogens, Science, 325 (2009) 1089-1093.

[3] K. Lewis, Platforms for antibiotic discovery, Nature Reviews Drug Discovery, 12 (2013) 371-387.

[4] L.L. Maragakis, T.M. Perl, Acinetobacter baumannii: Epidemiology, antimicrobial resistance, and treatment options, Clinical Infectious Diseases, 46 (2008) 1254-1263.

[5] K.K. Kumarasamy, M.A. Toleman, T.R. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C.G. Giske, S. Irfan, Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study, The Lancet infectious diseases, 10 (2010) 597-602.

[6] M.L. Cristina, A.M. Spagnolo, P. Orlando, F. Perdelli, The role of the environment in the spread of emerging pathogens in at-risk hospital wards, Reviews in Medical Microbiology, 24 (2013) 104-112.

[7] D.E. Karageorgopoulos, M.E. Falagas, Current control and treatment of multidrug-resistant Acinetobacter baumannii infections, Lancet Infectious Diseases, 8 (2008) 751-762.

[8] C. Nathan, Fresh Approaches to Anti-Infective Therapies, Science Translational Medicine, 4 (2012) 140sr142.

[9] A. MacGowan, M. Albur, Frontline antibiotic therapy, Clinical Medicine, 13 (2013) 263-268.

[10] A.M. Coates, G. Halls, Antibiotics in Phase II and III Clinical Trials, in: A.R.M. Coates (Ed.) Antibiotic Resistance, Springer Berlin Heidelberg2012, pp. 167-183.

[11] L.M. de Almeida, M.R.E. de Araújo, M.F. Iwasaki, A.G. Sacramento, D. Rocha, L.P. da Silva, M. Pavez, A.C. de Brito, L.C.S. Ito, A.C. Gales, N. Lincopan, J.L.M. Sampaio, E.M. Mamizuka, Linezolid resistance in vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolates in a Brazilian hospital, Antimicrobial Agents and Chemotherapy, 58 (2014) 2993-2994.

[12] B.P. Howden, J.K. Davies, P.D.R. Johnson, T.P. Stinear, M.L. Grayson, Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and

heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications, Clinical Microbiology Reviews, 23 (2010) 99-139.

[13] H. Tiwari, M. Sen, Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India, BMC Infectious Diseases, 6 (2006) 156.

[14] F.R. DeLeo, H.F. Chambers, Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era, The Journal of clinical investigation, 119 (2009) 2464-2474.

[15] R. Köck, K. Becker, B. Cookson, J. van Gemert-Pijnen, S. Harbarth, J. Kluytmans, M. Mielke, G. Peters, R. Skov, M. Struelens, Methicillin-resistant Staphylococcus aureus (MRSA): burden of disease and control challenges in Europe, (2010).

[16] B. Hamad, The antibiotics market, Nat Rev Drug Discov, 9 (2010) 675-676.

[17] L.L. Silver, Challenges of antibacterial discovery, Clinical microbiology reviews, 24 (2011) 71-109.

[18] D. Bruhn, D.B. Madhura, M. Maddox, R.B. Lee, A. Trivedi, L. Yang, M.S. Scherman, J.C. Gilliland, V. Gruppo, M.R. McNeil, Antitubercular nitrofuran isoxazolines with improved pharmacokinetic properties, Bioorganic & medicinal chemistry, 20 (2012) 6063-6072.

[19] D. Sun, R.B. Lee, R.P. Tangallapally, R.E. Lee, Synthesis, optimization and structure–activity relationships of 3, 5-disubstituted isoxazolines as new anti-tuberculosis agents, European journal of medicinal chemistry, 44 (2009) 460-472.

[20] R.P. Tangallapally, D. Sun, N. Budha, R.E. Lee, A.J. Lenaerts, B. Meibohm, R.E. Lee, Discovery of novel isoxazolines as anti-tuberculosis agents, Bioorganic & medicinal chemistry letters, 17 (2007) 6638-6642.

[21] R. Tiwari, P.A. Miller, S. Cho, S.G. Franzblau, M.J. Miller, Syntheses and Antituberculosis Activity of 1, 3-Benzothiazinone Sulfoxide and Sulfone Derived from BTZ043, ACS medicinal chemistry letters, 6 (2014) 128-133.

[22] CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically M07-A9, CLSI, Wayne, PA, 2012.

[23] A.S. Guram, R.A. Rennels, S.L. Buchwald, A simple catalytic method for the conversion of aryl bromides to arylamines, Angewandte Chemie International Edition in English, 34 (1995) 1348-1350.

[24] M.A. Vela, H. Kohn, Observations concerning the reactivity of bicyclomycin and bicyclomycin derivatives with organophosphorus reagents, The Journal of Organic Chemistry, 57 (1992) 6650-6653.

[25] R. Huisgen, Kinetics and Mechanism of 1,3-Dipolar Cycloadditions, Angewandte Chemie International Edition in English, 2 (1963) 633-645.

[26] M.E. Wand, J.W.I. McCowen, P.G. Nugent, J.M. Sutton, Complex interactions of Klebsiella pneumoniae with the host immune system in a Galleria mellonella infection model, Journal of Medical Microbiology, 62 (2013) 1790-1798.

[27] M.T. Holden, E.J. Feil, J.A. Lindsay, S.J. Peacock, N.P. Day, M.C. Enright, T.J. Foster, C.E. Moore, L. Hurst, R. Atkin, Complete genomes of two clinical Staphylococcus aureus strains: evidence for the rapid evolution of virulence and drug resistance, Proceedings of the National Academy of Sciences of the United States of America, 101 (2004) 9786-9791.

[28] G. Malich, B. Markovic, C. Winder, The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines, Toxicology, 124 (1997) 179-192.

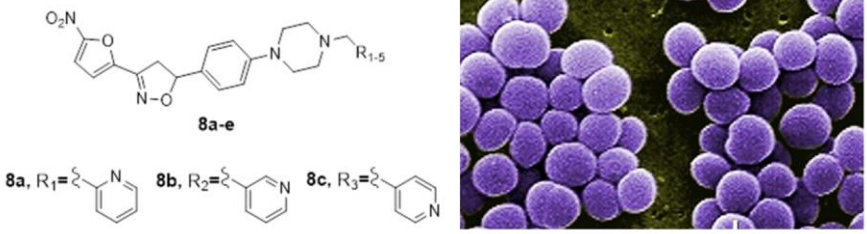
[29] V. Makarov, G. Manina, K. Mikusova, U. Möllmann, O. Ryabova, B. Saint-Joanis, N. Dhar, M.R. Pasca, S. Buroni, A.P. Lucarelli, A. Milano, E. De Rossi, M. Belanova, A. Bobovska, P. Dianiskova, J. Kordulakova, C. Sala, E. Fullam, P. Schneider, J.D. McKinney, P. Brodin, T. Christophe, S. Waddell, P. Butcher, J. Albrethsen, I. Rosenkrands, R. Brosch, V. Nandi, S. Bharath, S. Gaonkar, R.K. Shandil, V. Balasubramanian, T. Balganesh, S. Tyagi, J. Grosset, G. Riccardi, S.T. Cole, Benzothiazinones Kill Mycobacterium tuberculosis by Blocking Arabinan Synthesis, Science, 324 (2009) 801-804.

[30] A. Lannes, B. Leal, J. Novais, V. Lione, G.T.S. Monteiro, A. Lourenço, P. Sathler, A. Jordão, C. Rodrigues, L. Cabral, A. Cunha, V. Campos, V. Ferreira, M.V. de Souza, D. Santos, H. Castro, Exploring N-Acylhydrazone Derivatives Against Clinical Resistant Bacterial Strains, Current Microbiology, 69 (2014) 357-364.

[31] R.R. Zorzi, S.D. Jorge, F. Palace-Berl, K.F.M. Pasqualoto, L. de Sá Bortolozzo, A.M. de Castro Siqueira, L.C. Tavares, Exploring 5-nitrofuran derivatives against nosocomial pathogens: Synthesis, antimicrobial activity and chemometric analysis, Bioorganic & medicinal chemistry, 22 (2014) 2844-2854.

[32] I.D.S.o. America, Combating Antimicrobial Resistance: Policy Recommendations to Save Lives, Clinical Infectious Diseases, 52 (2011) S397-S428.

[33] J.S. Bradley, Which antibiotic for resistant Gram-positives, and why?, Journal of Infection, 68, Supplement 1 (2014) S63-S75.



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