Combining amoxicillin and relebactam provides new therapeutic potential for *Mycobacterium abscessus* infection

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Key Words:

Mycobacterium abscessus; Non-tuberculous mycobacteria; Antimicrobial drug discovery; Cystic fibrosis; β-lactam; β-lactamase; synergy

Abstract:

Infections caused by the opportunistic pathogen *Mycobacterium abscessus* are increasing in prevalence within the cystic fibrosis population. Our limited understanding of this ubiquitous environmental microorganism matched with its intrinsic resistance to most classes of antibiotic, including β-lactams, has left us with an urgent demand for new, more effective therapeutic interventions. *De novo* antimicrobial drug discovery is a lengthy process and so we have taken the approach of repurposing known antibiotics in order to provide a rapidly implementable solution to a current problem. Here we report a significant step forward in treatment potential for *M. abscessus* infection by sensitising the organism to the broad spectrum β-lactam antibiotic, amoxicillin, using the non-competitive β-lactamase inhibitor, relebactam. We demonstrate by disk diffusion and broth microdilution assay that this combination works synergistically to inhibit *M. abscessus.* We also demonstrate the direct non-competitive inhibition of the *M. abscessus* β-lactamase, Bla_{Mab} using a novel thin-layer chromatography-based assay for β-lactamase inhibition, which is subsequently kinetically validated by spectrophotometric assay using the nitrocefin reporter assay. Finally, we demonstrate the *in vitro* efficacy of this combination against a collection of *M. abscessus* clinical isolates, demonstrating the significant therapeutic potential of the amoxicillin and relebactam combination.

Introduction:

The incidence of infections with the rapidly growing non-tuberculous mycobacterial (NTM) organism *Mycobacterium abscessus* is increasing due to the increase of immunocompromised individuals (Petrini, et al. 2006) (Griffith, et al. 2014). *M. abscessus* is widely responsible for opportunistic pulmonary infections in patients with structural lung disorders such as cystic fibrosis (CF) and bronchiectasis (Griffith, et al., 2007) as well as skin and soft tissue infections (SSTIs) in humans (Moore, 1953) (Fitzgerald, et al., 1995). The ubiquitous environmental nature of *M. abscessus* may go some

47 way to explaining the high levels of intrinsic drug resistance to most major classes of antibiotic that is
48 observed clinically (Nessar, et al. 2012). Recently, macrolides were added to the ever growing list of
49 ineffective antimicrobial agents for *M. abscessus* infection, exemplifying the urgent need for new ways
50 to treat this infection (Nessar, et al. 2012). Additionally, *M. abscessus* is resistant to two of the
51 frontline antibiotics used for tuberculosis treatment; rifampicin and ethambutol (Nessar, et al. 2012),
52 and many of the new drugs being discovered for treating tuberculosis do not exhibit any antimicrobial
53 activity against *M. abscessus* (Soni, et al. 2016).

54

55 Pulmonary *M. abscessus* disease was historically treated with macrolide antibiotics such as
56 clarithromycin (Griffith, et al., 2007), however the steady emergence of antibiotic resistant strains has
57 prompted the implementation of combination therapy using azithromycin (macrolide) along with
58 amikacin (aminoglycoside) and at least one other drug of a different class (Leung, et al. 2013). The
59 British Thoracic Society recommends a biphasic treatment for pulmonary *M. abscessus* infection
60 comprising of a 1-month initiation phase including intravenously administered amikacin, tigecycline
61 and imipenem, supplemented with oral clarithromycin or azithromycin (depending on sensitivity
62 results). This is followed by a 12-month continuation phase comprising of nebulised amikacin, and a
63 combination of 1-4 of clofazimine, linezolid, minocycline, moxifloxacin or co-trimoxaole (Lopeman et
64 al. 2019) (Haworth et al 2017).

65

66 The advancements in whole genome sequencing technology have enabled the identification of three
67 distinct subspecies within the *M. abscessus* complex, comprising *M. abscessus* subsp. *abscessus, M.*
68 *abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense,* the latter of which contains a non-
69 functional *erm*(41) gene, resulting in a macrolide sensitive phenotype (Bryant et al. 2013) (Tortoli et
70 al. 2016). Mutations in *rrs* and *rrl* 16S rRNA genes have been identified corresponding to high levels of
71 aminoglycoside resistance, prohibiting the effective use of amikacin in treatment for *M. abscessus*

72 subsp. a*bscessus* and *M. abscessus* subsp. m*assiliense* clinical isolates specifically (Kehrmann et al.
73 2016). All *M. abscessus* subsp. contain an endogenous class A β-lactamase (Bla_{Mab}) conveying intrinsic
74 resistance to the β-lactam antibiotics (Soroka et al 2014). The identification of Bla_{Mab}, a homolog of
75 the *Mycobacterium tuberculosis* endogenous β-lactamase, BlaC, sparked widespread investigation
76 into the potential of β-lactamase inhibitors to supplement the treatment of *M. abscessus* infection. A
77 study demonstrated the inhibitory activity of avibactam, a non-β-lactam β-lactamase inhibitor,
78 against Bla_{Mab} significantly improving the *in vitro* and *in vivo* activity of imipenem (Lefebvre et al. 2016).
79 Very recently, a number of studies have been published demonstrating a number of β-lactam
80 combinations displaying high levels of *in vitro* synergistic activity against *M. abscessus* complex
81 (Pandey et al 2019) (Story-Roller et al. 2019). Furthermore, a recent study identified the *in vitro* activity
82 of two new non-β-lactam β-lactamase inhibitors, namely relebactam and vaborbactam,
83 demonstrating synergistic activity between imipenem and relebactam as well as meropenem and
84 vaborbactam (Kaushik et al. 2019).

85

86 In this study, we have built on previous work and identified a significant step forward in treatment
87 potential for *M. abscessus* infection. We have demonstrated the *in vitro* capacity for sensitising the
88 organism to the broad spectrum β-lactam antibiotic, amoxicillin, using the non-competitive β-
89 lactamase inhibitor, relebactam. We have demonstrated direct, non-competitive inhibition of
90 recombinant Bla_{Mab} by relebactam, and demonstrated synergy between amoxicillin and relebactam as
91 well as activity against a range of clinical isolates. This offers a considerable therapeutic potential as
92 imipenem, a mainstay of current *M. abscessus* treatment, is currently undergoing phase III clinical trial
93 in combination with relebactam, suggesting the simple addition of amoxicillin and relebactam to the
94 treatment regime has the potential to significantly improve the treatment outcome.

95

96

Materials and Methods:

Bacterial isolates

A total of 16 *M. abscessus* clinical isolates from Brighton and Sussex Medical School and *M. abscessus* NCTC 13031 were used in this study. Stock solutions of the isolates were kept in 50% glycerol (Sigma, Dorset, UK) and Middlebrook 7H9 Broth and stored at -80 °C. Isolates were grown in Middlebrook 7H9 medium supplemented with 10% oleic acid-dextrose-catalase (OADC), 1% glycerol (50% w/v) and 0.05% Tween 80 (v/v) prior to testing. *Escherichia coli* Top 10 cells were used for propagation of plasmid DNA. These cells were routinely grown in nutrient broth, or nutrient agar (Oxoid, UK) at 37 °C. *E. coli* BL21 (DE3) cells were used for the overproduction of recombinant protein, grown in Terrific Broth (Melford, UK) at 37 °C.

Antimicrobials

The antimicrobial agents meropenem (MEM), amoxicillin (AMX) and phenoxymethylpenicillin (Penicillin V/PenV) were obtained from Sigma Aldrich (Dorset, UK) and relebactam was obtained from Carbosynth (Compton, UK). Stock solutions were prepared in sterile de-ionised water and stored at -20 °C until use.

Disk diffusions

For the disk diffusion assays, *M. abscessus* clinical isolates were grown in Middlebrook 7H9 Broth to logarithmic phase and 100 µL of bacterial culture was inoculated into 10 mL Middlebrook 7H9 Broth supplemented with 0.7% bacteriological agar. This was poured as a layer in agar plates on top of 15 mL Middlebrook 7H11 Agar supplemented with 10% OADC and 1% glycerol. 6 mm sterile filter paper

120 diffusion disks were placed on the agar and were subsequently impregnated with antibiotic at the
121 following concentrations in sterile distilled water: relebactam 1 µL of 10 mg/mL, amoxicillin 3.3 µL of
122 3 mg/mL and meropenem 1 µL of 10 mg/mL. Plates were incubated at 30 °C for 5 days or until clear
123 zones of inhibition were visualised. Zones of inhibition were measured across the diameter to include
124 the disk itself.

125

126 **Broth microdilution assay**

127 The broth microdilution assay was performed as described previously with alterations appropriate to
128 this study (Institute, Clinican and Laboratory Standards 2012) (Caleffi-Ferracioli et al. 2013). Plates
129 were prepared by serially diluting AMX (0.128 mg/mL – 0 mg/mL) or MEM (0.128 mg/mL – 0 mg/mL)
130 in the *x*-axis and REL (0.128 mg/mL – 0 mg/mL) in the *y*-axis. 80 µL of *Mycobacterium abscessus* NCTC
131 13031 suspension adjusted to an OD_{600} of 0.1-0.2 was inoculated in each well to reach a final well
132 volume of 100 µL. Plates were sealed and incubated at 30 °C for 5 days or until positive control wells
133 reached an OD_{600} of 1.0. The MIC of amoxicillin/relebactam combination was determined by
134 visualising the well that had the lowest concentrations of both compounds and exhibited no bacterial
135 growth. The relevant well was retroactively plotted into a growth curve over time, and this growth
136 curve was compared to the wells containing no drug, and the wells containing relebactam only and
137 amoxicillin only.

138

139 **Expression and purification of recombinant Bla_{Mab}**

140 The *bla_{Mab}* gene (MAB_2875) from *Mycobacterium abscessus* NCTC 13031 was amplified by
141 polymerase chain reaction (PCR), with the first 90 base pairs omitted (resulting in a -30 residue N-
142 terminal truncated protein). Primers used were as follows (with restriction site underlined): Forward
143 primer AAAAAA<u>GGATCC</u>GCGCCGGACGAACTCGCC and Reverse primer

AAAAAAAAGCTTTCAAGCGCCGAAGGCCCG (Eurofins Genomics). Amplicons were purified and cloned into pET28a using *Bam*HI/*Hin*DIII restriction sites and the sequence was confirmed (Eurofins Genomics). Bla_{Mab} was expressed in *E. coli* BL21 (DE3) cells by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 25 °C for 18 h. Bla_{Mab} was purified by Immobilised Metal Affinity Chromatography (IMAC) and dialysed into 25 mM Tris HCl pH 7, 100 mM NaCl.

Thin Layer Chromatography (TLC) activity assay

Relebactam (2 mg/mL) was added to recombinant Bla_{Mab} (0.01 mg/mL) and incubated for 5 minutes at room temperature, before addition of Penicillin V (4 mg/mL) for a further 10 minute incubation at room temperature. Alongside appropriate control reactions (Figure 2), 1 µL of the reaction was spotted onto aluminium backed silica gel plates (5735 silica gel 60 F_{254}, Merck) and dried before being subjected to Thin Layer Chromatography (TLC) using ethyl acetate:water:acetic acid ($C_4H_8O_2$:H_2O:CH_3COOH) (3:1:1, v/v/v). Once dry, plates were visualised by being dipped into $KMnO_4$ TLC stain with light charring.

Spectrophotometric analysis of Bla_{Mab} inhibition by Relebactam

Recombinant Bla_{Mab} (0.25 nM) was mixed with an increasing concentration of relebactam (0, 0.1, 1, 10 and 100 µM) and 100 µM nitrocefin (Carbosynth, UK). The hydrolysis of nitrocefin was monitored at 486 nm using a Multiskan Go plate reader (Thermo Scientific). Data points were subsequently plotted as absorbance at 486 nm vs time using Graphpad Prism 7.

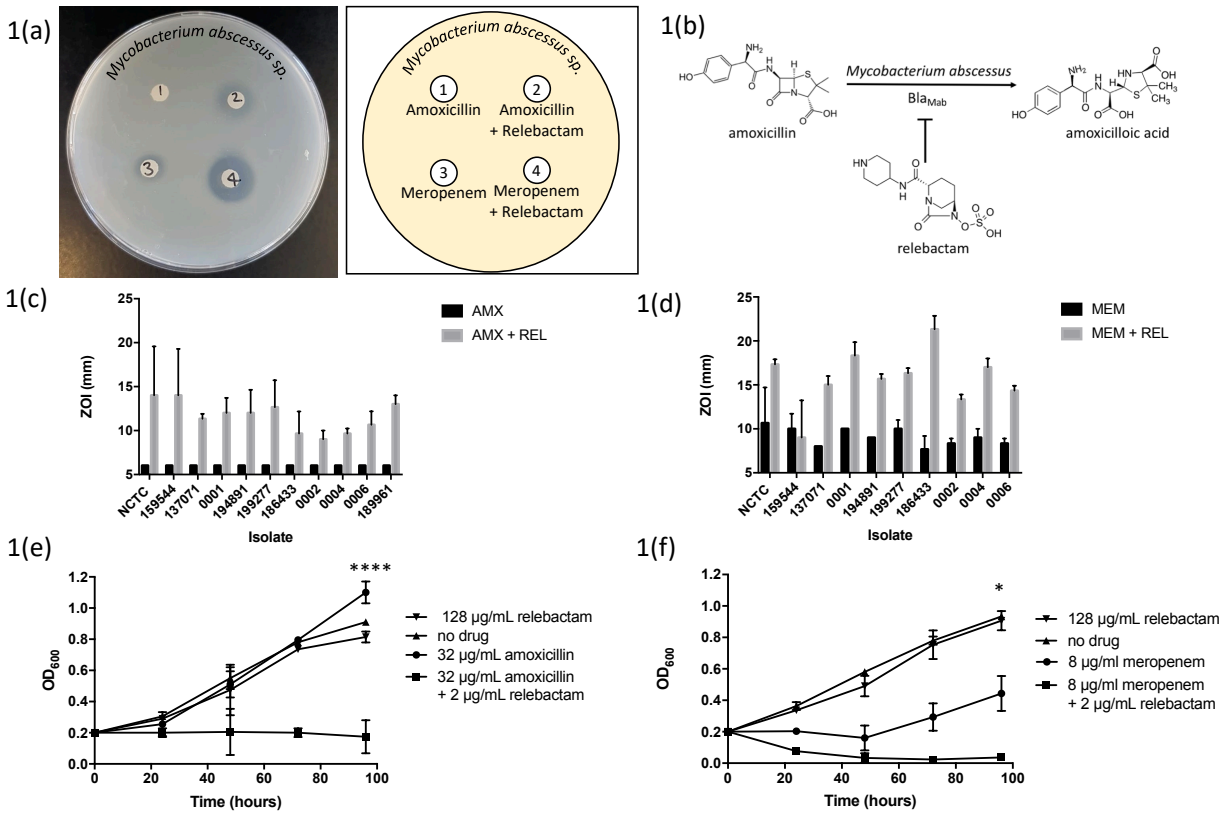
Results:

In this paper we have identified that *M. abscessus* can be sensitised to amoxicillin by the addition of the non-competitive β-lactamase inhibitor, relebactam. Furthermore we have shown that meropenem sensitivity can be enhanced by the addition of relebactam. Firstly, we conducted disk diffusion assays with amoxicillin and meropenem with and without relebactam. Subsequently the zones of inhibition (ZOI) were measured as a measure of sensitivity. Amoxicillin alone failed to demonstrate any kind of sensitivity, however the addition of relebactam provided detectable ZOI, demonstrating the induced sensitivity to amoxicillin by relebactam. A ZOI was visible for meropenem, however with the incorporation of relebactam, this ZOI was significantly enhanced, suggesting increased sensitivity upon combination with relebactam (1a). This led us to the hypothesis that relebactam was inhibiting the *M. abscessus* β-lactamase, Bla_{Mab}, preventing hydrolysis of amoxicillin to the inactive amoxicilloic acid (1b). In order to assess the clinical relevance of these observations, both amoxicillin and meropenem were tested alone and in combination with relebactam against a panel of clinical isolates of *M. abscessus* obtained from patients at Brighton and Sussex Medical School in addition to *M. abscessus* NCTC strain. In all cases, the addition of relebactam sensitised the isolate to amoxicillin (*n=3*) (1c). The same experiment was conducted with meropenem, and in most cases, an increase in sensitivity was observed (*n=3*) (1d). All necessary controls were conducted and no ZOI was observed for relebactam alone.

In order to further validate our observation, liquid cultures were exposed to increasing concentrations of amoxicillin and meropenem with and without a dose response of relebactam (1e and 1f respectively). As a control, relebactam was also tested for inhibitory activity on its own. These cultures were read every 24 hours using a spectrophotometer at OD_{600nm} up to 96 hours. Relebactam at 128 µg/mL demonstrated no inhibitory activity, giving a growth profile much the same as the compound-free control (no drug), confirming that relebactam lacks antibacterial activity against *M. abscessus*.

192 32 µg/mL of amoxicillin appeared to show a moderate enhancement of bacterial growth, however
193 when combined with 2 µg/mL of relebactam, this amoxicillin concentration was found to display
194 potent antibacterial activity against *M. abscessus*. The same experiment was conducted with
195 meropenem, wherein partial growth impairment was observed at 8 µg/mL, however when combined
196 with 2 µg/mL relebactam, significant antibacterial activity was observed at this concentration. End
197 point statistical analysis was conducted using a t-test to assess the significance of differences between
198 cultures with and without relebactam. In both cases, a statistically significant increase in sensitivity
199 was observed upon addition of 2 µg/mL of relebactam in combination with amoxicillin or meropenem.

200



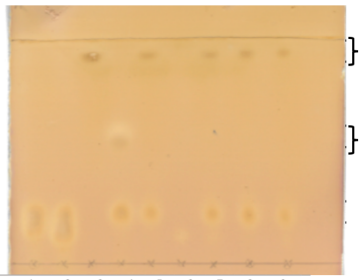
201

202 **Figure 1: Relebactam sensitises *Mycobacterium abscessus* to amoxicillin and improves sensitivity to**
203 **meropenem.** A disk diffusion experiment (1a) and corresponding plate map demonstrating enhanced
204 sensitivity of *M. abscessus* by zone of inhibition to amoxicillin (1) with the addition of relebactam (2)
205 and meropenem (3) with the addition of relebactam (4). The inhibition of *M. abscessus* β-lactamase

206 Bla_{Mab} by relebactam is summarised (1b). The disk diffusion experiments were conducted with the
207 NCTC *M. abscessus* strain along with a panel of clinical isolates and the diameter of the zones of
208 inhibition (ZOI) measured in millimetres. All strains of *M. abscessus* tested were resistant to amoxicillin
209 (AMX) alone, however displayed sensitivity with the addition of relebactam (REL) (1c). The same was
210 conducted for meropenem (MEM) and likewise, activity was clearly enhanced with the addition of
211 relebactam (1d). Growth curves were conducted with *M. abscessus* NCTC in medium containing
212 128 $\mu g/mL$ relebactam and 32 $\mu g/mL$ amoxicillin with and without relebactam at 2 $\mu g/mL$. Growth
213 inhibition was only observed with relebactam in combination with amoxicillin (1e). Likewise, the
214 inhibitory activity of meropenem was clearly enhanced with the addition of relebactam (1f). A t-test
215 was used for end point analysis between samples +/- relebactam and the results were deemed to be
216 significant with *p* values of <0.0001 and 0.0152 for amoxicillin and meropenem respectively.

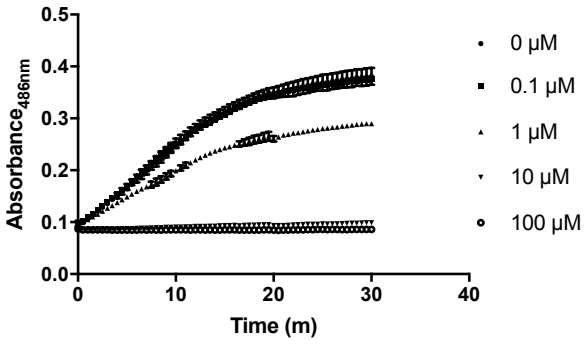
217

2(a)

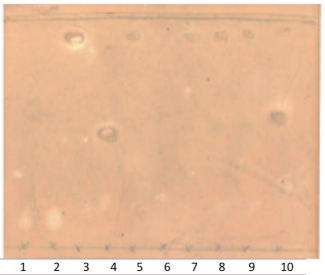


	1	2	3	4	5	6	7	8	9
Buffer	X	X	X	X	X	X	X	X	X
Penicillin V			X	X	X		X	X	X
BlaMab		X		X	X		X		
Relebactam						X	X		X
Boiled BlaMab								X	X
Avibactam					X				

2(b)

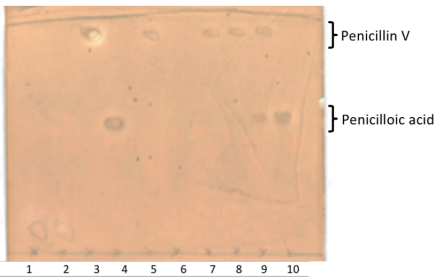


2(c)



	1	2	3	4	5	6	7	8	9	10
Buffer	X	X		X	X		X	X	X	X
Penicillin V			X	X	X		X	X	X	X
Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Relebactam 0s						X				X
Relebactam 10s							X			
Relebactam 30s								X		
Relebactam 60s									X	

2(d)



	1	2	3	4	5	6	7	8	9	10
Buffer	X	X		X	X		X	X	X	X
Penicillin V			X	X	X		X	X	X	X
Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Relebactam 200 µg/mL						X	X			
Relebactam 20 µg/mL								X		
Relebactam 2 µg/mL									X	
Relebactam 0.2 µg/mL										X

218

Figure 2. Biochemical analysis of relebactam inhibition of *M. abscessus* β-lactamase, Bla_{Mab}. Our novel Thin Layer Chromatography (TLC) assay (2a) exhibiting the activity of Bla_{Mab} in the turnover of penicillin V (high R_f value) to penicilloic acid (lower R_f value). In the absence, or termination of activity of Bla_{Mab} (by boiling (100 °C for 1 h) or addition of known inhibitor avibactam (Lefebvre et al., 2017) (200 µg/mL) no lower R_f value spot corresponding to penicilloic acid is seen on the TLC plate. The addition of relebactam to the reaction between Bla_{Mab} and penicillin V also results in the absence of the lower Rf value spot, suggesting inhibition of Bla_{Mab} (2a). This observed inhibition is validated by a spectrophotometric analysis, where the turnover of nitrocefin (100 µM) by Bla_{Mab} (0.25 nM) was monitored at 486 nm with a varying concentration of relebactam (0, 0.1, 1, 10 and 100 µM). The increase in concentration of relebactam resulted in partial inhibition of nitrocefin turnover at 1 µM and total abrogation at 10 µM (2b). This inhibitory activity was seen within 10 seconds of pre-incubation of relebactam with Bla_{Mab}, before addition of penicillin V, in a time course TLC assay. However, the addition of relebactam at the same time (t=0) as penicillin V resulted in a lack of inhibitory activity (2c). The minimum concentration of relebactam required for inhibition of Bla_{Mab} in the TLC activity assay was assessed using a range of concentrations (200, 20, 2 and 0.2 µg/mL). Activity of Bla_{Mab} was maintained below a relebactam concentration of 2 µg/mL in the TLC activity assay, suggesting a minimal concentration of relebactam required to inhibit Bla_{Mab} in the assay is within the range of 20 to 2 µg/mL (2d).

In order to validate the inhibitory activity observed phenotypically in Figure 1, we conducted biochemical analysis of the activity of relebactam on the *M. abscessus* endogenous β-lactamase, Bla_{Mab}. The gene was amplified by PCR, digested, ligated into pET28a and sequenced, before transformation into chemically competent *E. coli* BL21. Bla_{Mab} was expressed, cells harvested and the enzyme purified by IMAC. We subsequently devised a novel TLC-based assay for assessing β-lactamase activity by separating the penicillin V substrate from the penicilloic acid product. This assay

244 enabled us to demonstrate the β-lactamase activity of our purified Bla_{Mab}, as well as assay the efficacy
245 of inhibitors against the enzyme (2a). We used avibactam as a positive control, as its inhibitory activity
246 against Bla_{Mab} has previously been described by Lefebvre et al. 2017. Lane 1 contained protein
247 purification buffer only, and lane 2 had the addition of enzyme (0.01 mg/ml). Following
248 chromatography, two lower spots are observed in lanes 1 and 2, indicative of buffer. Penicillin V was
249 added to lane 3 and gave a characteristic spot of high R_f value, demonstrating unhydrolysed penicillin
250 resulted in a spot just below the solvent front. Lane 4 was identical to lane 3 with the addition of Bla_{Mab}
251 protein. The hydrolysis of penicillin V to penicilloic acid by Bla_{Mab} resulted in a spot with reduced R_f
252 value. The pre-incubation of enzyme with avibactam in lane 5 resulted in a loss of the lower penicilloic
253 acid spot, demonstrating the inhibition of Bla_{Mab} activity. In lane 6, relebactam alone did not resolve
254 on the TLC, but its pre-incubation with Bla_{Mab} before addition to the penicillin substrate in lane 7
255 resulted in total inhibition of hydrolysis as observed with avibactam (lane 5). Finally, the inhibition of
256 Bla_{Mab} is further validated by the repeat of lane 4 and lane 7 conditions with heat-denatured Bla_{Mab}
257 (lanes 8 and 9 respectively). This result confirms the direct inhibition of Bla_{Mab} by relebactam (*n=5*).

258

259 We further validated this result by using a commercially available β-lactamase substrate, nitrocefin,
260 which is selectively hydrolysed by β-lactamases, resulting in an increase in absorbance at 486 nm. By
261 pre-incubating Bla_{Mab} enzyme with a dose response of relebactam (from 0-100 µM), before initiation
262 of the absorbance assay with the addition of nitrocefin substrate, we observed partial inhibition at
263 1 µM (0.348 µg/mL) and a complete loss of activity at 10 µM (3.48 µg/mL), confirming the direct
264 inhibitory activity of relebactam on Bla_{Mab} (2b).

265

266 Our TLC-based β-lactamase assay enabled us to further explore the parameters of the inhibitory
267 activity of relebactam by varying the time of pre-incubation of relebactam with Bla_{Mab} (2c) and the
268 minimum inhibitory concentration required to abrogate catalytic turnover of the penicillin V substrate

to the penicilloic acid substrate (2d). We found that penicillin V turnover was rapid and that only addition of relebactam at the same time as the substrate demonstrated turnover of penicillin V, suggesting non-competitive, irreversible inhibition of Bla_{Mab} by relebactam. The dose response of relebactam demonstrated total inhibition down to 20 µg/mL, and activity of Bla_{Mab} was maintained below a relebactam concentration of 2 µg/mL. This suggested a minimal concentration of relebactam required to inhibit Bla_{Mab} in the assay is within the range of 20 to 2 µg/mL. This corresponds to less than or equal to 100 fold stoichiometric excess of relebactam required to completely inhibit Bla_{Mab} (0.5 µM BlaMab to 57.5 µM relebactam corresponding to 20 µg/mL).

Discussion:

In this paper we have demonstrated the inhibitory activity of the non-β-lactam based β-lactamase inhibitor, relebactam against *M. abscessus* endogenous β-lactamase, Bla_{Mab}. Alone, this is not sufficient to kill *M. abscessus,* however its use as part of a combination opens up considerable therapeutic potential. We have identified that a low concentration of relebactam co-administration is capable of sensitising *M. abscessus* NCTC and clinical isolates to amoxicillin, well within the therapeutic range for this versatile and widely available antibiotic. Furthermore, we demonstrate relebactam provides a significant increase in inhibitory activity of meropenem, a mainstay of *M. abscessus* clinical intervention. Our study introduces a completely novel TLC-based β-lactamase inhibition assay, validated with the commercially available and widely used nitrocefin assay, to investigate the parameters of relebactam's inhibitory activity. Relebactam is currently in phase III clinical trial for administration in combination with imipenem, another mainstay in *M. abscessus* front line chemotherapy. Our findings therefore represent a timely and highly impactful discovery that is likely to be translatable into the clinical setting.

294 **Author Contributions:**

295 R. C. L., J. H., M. D., P. L. and J. A. G. C. intellectually conceived and designed the experiments. R. C. L.,
296 J. H. and J. A. G. C. conducted the experiments. R. C. L., J. H. and J. A. G. C. wrote the manuscript.

297

298 **Funding:** This research was funded by Birmingham Women's and Children's Hospital Charity Research
299 Foundation (BWCHCRF) (R. C. L. 50% PhD Studentship, match funded by Aston University Prize
300 Scheme) and the Academy of Medical Sciences and Global Challenges Research Fund with a
301 Springboard Grant (SBF003\1088:).

302

303 **Acknowledgements:** J. A. G. C. is grateful to the Academy of Medical Sciences, Global Challenges
304 Research Fund and Birmingham Women's and Children's Hospital Charity Research Foundation
305 (BWCHCRF) for their continued support of the Mycobacterial Research Group at Aston University. The
306 *Mycobacterium abscessus* clinical isolates used in this study were provided by Dr Simon Waddell,
307 Brighton and Sussex Medical School.

308

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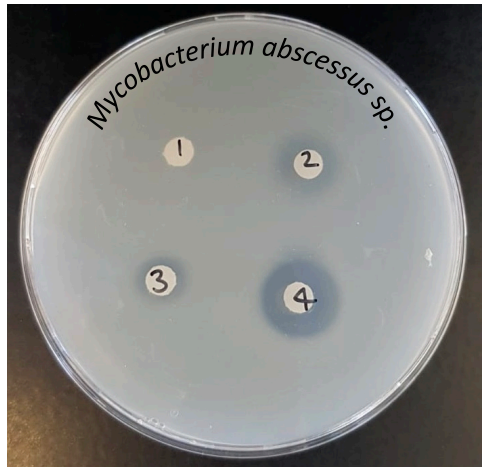

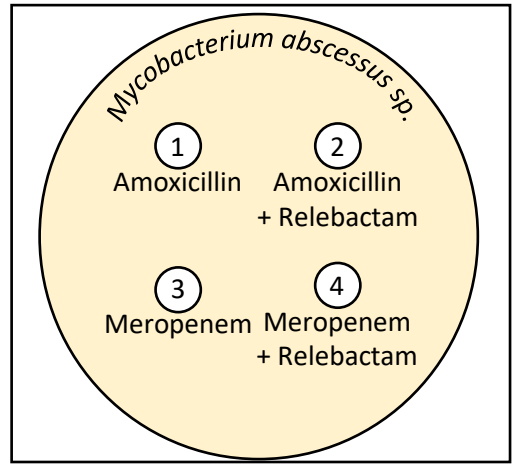

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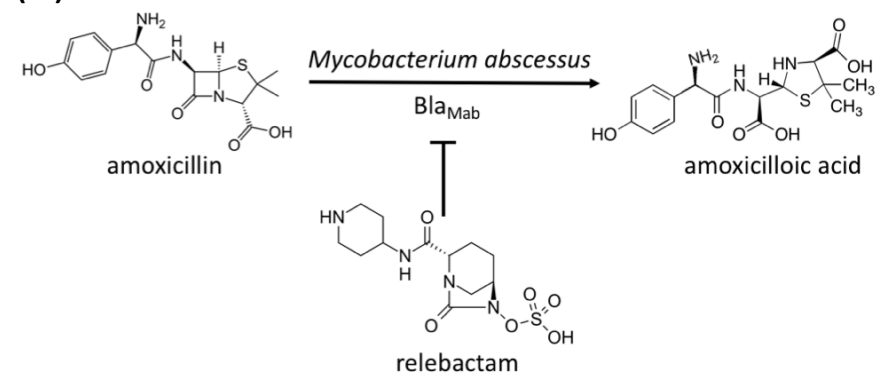

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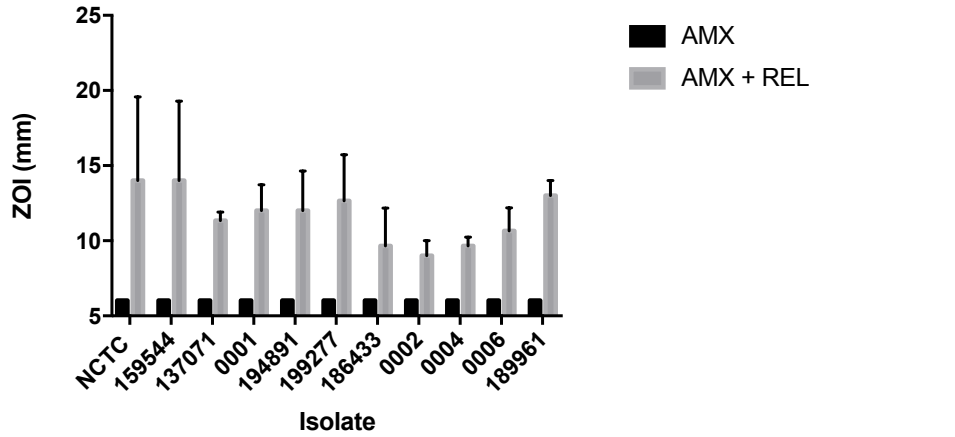

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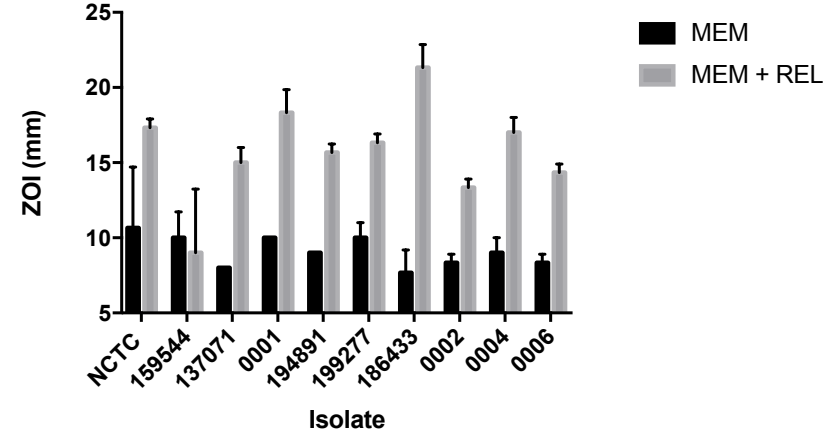

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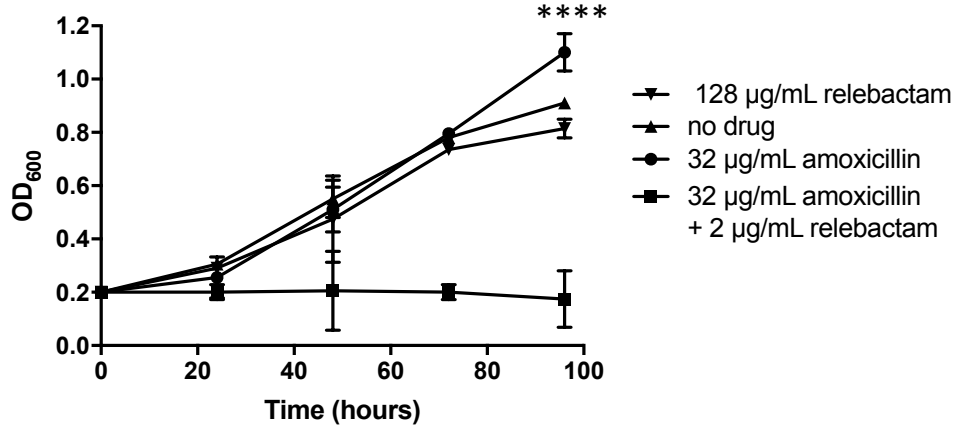

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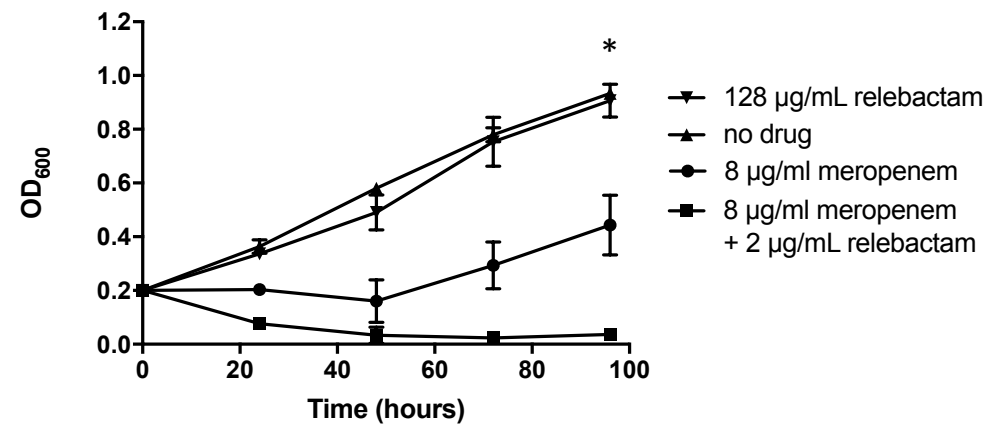

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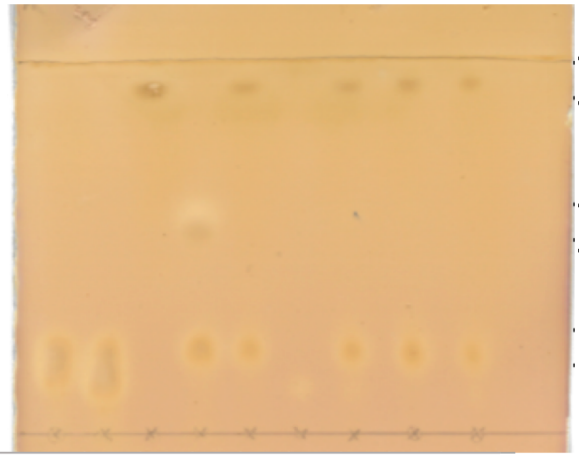

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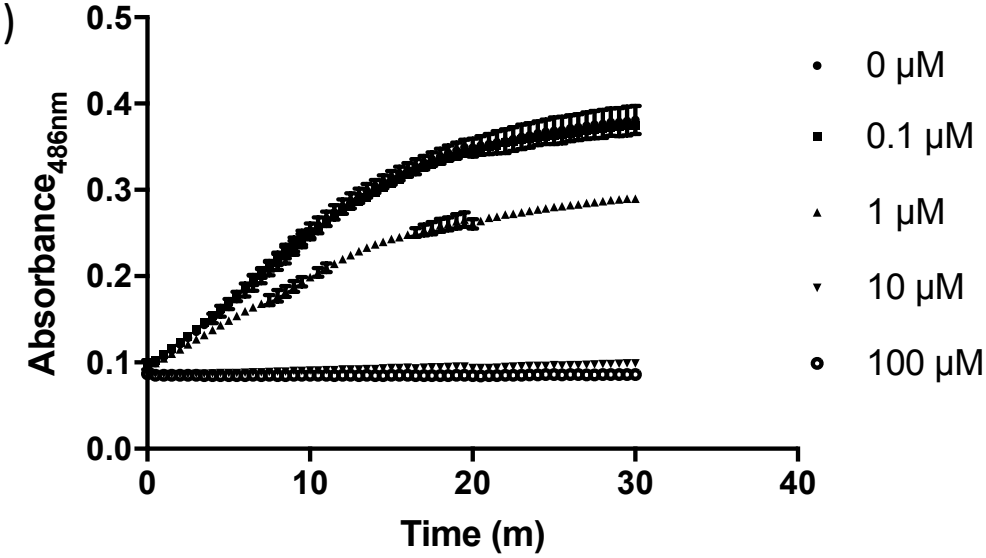
2(b)



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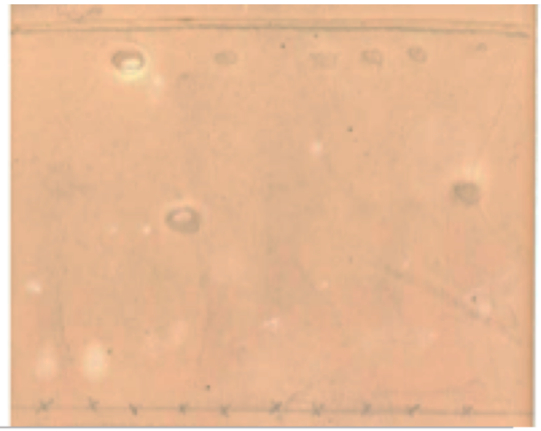

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Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Relebactam 0s						X				X
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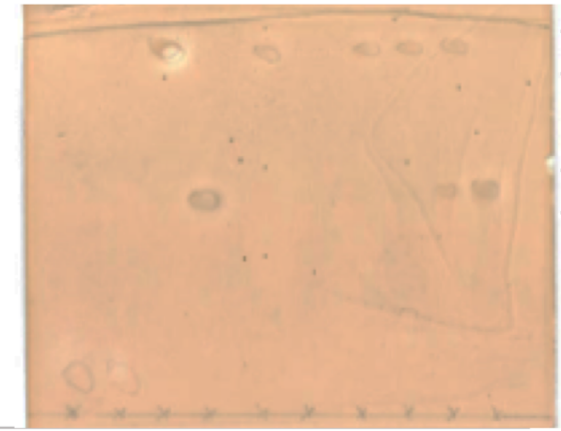
Relebactam 60s									X	

2(d)



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Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Relebactam 200 µg/mL						X	X			
Relebactam 20 µg/mL								X		
Relebactam 2 µg/mL									X	
Relebactam 0.2 µg/mL										X