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1 # Mechanistic and phenotypic studies of bicarinalin,

2 # BP100 and colistin action on *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii has been identified by the WHO as a high priority pathogen. It can be resistant to multiple antibiotics and colistin sulphate is often used as a last-resort treatment. However, the potentially severe side-effects of colistin are well documented and this study compared the bactericidal and anti-biofilm activity of two synthetic nature-inspired antimicrobial peptides, bicarinalin and BP100, with colistin. The minimum bactericidal concentration (MBC) against planktonic *A. baumannii* was approximately 0.5 μg/ml for colistin sulphate and ~4 μg/ml for bicarinalin and BP100. *A. baumannii* commonly occurs as a biofilm and biofilm removal assay results highlighted that both bicarinalin and BP100 had significantly greater potential than colistin. Atomic force microscopy (AFM) showed dramatic changes in *A. baumannii* cell size and surface conformity when treated with peptide concentrations at and above the MBC. Scanning electron microscopy (SEM) visualised the reduction of biofilm coverage and cell surface changes as peptide concentration increased. Liposome assays revealed that these peptides most likely act as pore-forming agents in the membrane. Bicarinalin and BP100 may be effective therapeutic alternatives to colistin against *A. baumannii* infections but further research is required to assess if they elicit cytotoxicity issues in patients.

Introduction

It has been widely publicised that by 2050, deaths due to antimicrobial resistant (AMR) infections may rise to 10 million per year and during that time, 300 million people will die from AMR infections [1]. The abuse of currently used antimicrobials, the paucity of new antimicrobials progressing successfully through clinical trials and the evolution of bacterial resistance mechanisms have led to the prospect of returning to the pre-antibiotic era when patients were dying from infections through minor injuries and routine surgeries. Although the recent development of novel antibiotics [2, 3] has provided reason for optimism, these drugs are generally limited to the treatment of Gram-positive infections while Gram-negative pathogens remain of significant concern [4].

Acinetobacter baumannii is one such opportunistic pathogen [5, 6] occurring almost exclusively in the hospital environment and is particularly prevalent in intensive care and burns units; it has been reported to be responsible for between 2-10% of all Gram-negative nosocomial infections [7]. A member of the 'ESKAPE' group [8] of pathogens, *A. baumannii* is able to persist on clinical surfaces by forming biofilms [7, 9], an ability that renders bacteria more resistant to many common antimicrobials [10, 11] leading to *A. baumannii* infections becoming rapidly more difficult to treat [12]. It is able to persist for long periods on fomites [7, 9] enabling the pathogen to cause widespread epidemic infections in nosocomial settings. Current treatments include β-lactam antibiotics with the carbapenems typically the treatment of choice [9].

Antimicrobial peptides (AMPs) have been much heralded as alternatives to antibiotics due to their ability to destroy multi-drug resistant (MDR) bacteria [13]. One example is colistin

60   sulphate (colistin), commonly used as a last resort treatment for MDR *A. baumannii* infections

61   [14]. However, the toxicity of colistin in patients is well known [13, 14] and resistance is

62   increasingly described [15-17] resulting in a crucial search for improved alternatives.

63   The aim of this study was to establish and visualise the activity of two little researched

64   AMPs, bicarinalin and BP100, which may be appropriate for use instead of colistin to treat MDR

65   *A. baumannii* infections. Bicarinalin is an amphipathic, C-terminally amidated, novel

66   antimicrobial peptide derived from the venom of the myrmicine ant, *Tetramorium bicarinatum*

67   [18, 19]. It consists of a sequence of 20 amino acid residues (KIKIPWGKVKDFLVGGMKAV) with a

68   molecular weight of 2213.78 g/mol. The action of bicarinalin has been evaluated against a

69   selection of Gram-positive and Gram-negative organisms [18, 19] and has been found to have

70   good antibacterial activity compared to other AMPs together with less haemolytic activity.

71   BP100 is a short C-amidated undecapeptide consisting of 11 amino acids (KKLFKKILKYL)

72   and a molecular weight of 1420.88 g/mol. It was originally synthesised by combinatorial

73   chemistry involving two peptides, cecropin A and melittin [20]. Cecropin A is a member of the

74   well-researched AMP family, the cecropins, first isolated from the giant silk moth *Hyalophora*

75   *cecropia*. The cecropin family, although susceptible to protease degradation, does not exhibit

76   cytotoxic effects against human erythrocytes [21, 22]. Melittin is a 26 amino acid, haemolytic

77   alpha helical peptide first purified from the European honeybee in 1958 [23], with

78   demonstrated antibacterial activity [24]. However, on its own, melittin's strong cytotoxic action

79   makes it unsuitable for clinical applications [25]. To circumvent cecropin A's susceptibility to

80   proteolytic degradation and melittin's high cytotoxicity, they were combined to produce a

81   derivative, BP100. BP100 exhibits low susceptibility to protease degradation and lower

82 cytotoxicity against erythrocytes and fibroblasts [26]. BP100 has been established to have good

83 antibacterial activity against several Gram-negative bacteria [20, 22].

84   It is believed that bicarinalin's antibacterial mechanism is similar to other AMPs, with its

85 cationic charge, as a result of lysine residues, naturally attracted to the anionic charged

86 bacterial cell surface. It is also believed that BP100 interacts with the bacterial cell membrane

87 via electrostatic attraction to the negatively charged LPS layer, causing blebbing on the surface

88 leading to a collapse of the outer membrane [20].

89   The aim of this study was to assess the potential of bicarinalin and BP100 as alternatives

90 to colistin to treat *A. baumannii* infections. The study also aimed to visualise the effects of these

91 antimicrobial agents on *A. baumannii* cells and biofilms using scanning electron microscopy

92 (SEM) and atomic force microscopy (AFM).

Materials and methods

Bacterial culture conditions

Fresh cultures of *Acinetobacter baumannii* (ATCC® 19606) were prepared by streaking a

culti-loop (ThermoFisher Scientific, UK) on freshly prepared Tryptone Soya Agar (TSA, Oxoid,

UK) and incubated for 24 h at 37 °C. Purity was assessed using Gram staining, cell morphology,

oxidase and Analytical Profile Index (API, Biomérieux, UK, 20NE kit) testing. A standard growth

curve for the *A. baumannii* was established to ensure mid-log phase growth and an initial

inoculum of $5x10^5$ CFU/ml at the start of each experiment.

Antimicrobial assays

Minimum inhibitory concentration (MIC)

The MIC was determined for each of the antimicrobial agents studied: colistin (Sigma-

Aldrich, UK), bicarinalin (97.7% purity, Genscript, USA) and BP100 (98.4% purity, Genscript,

USA). 10 ml of sterile Tryptone Soya Broth (TSB, Oxoid, UK) was inoculated with 2-3 colonies of

A. baumannii and incubated overnight at 37 °C and later diluted to an absorbance that equated

to $1x10^6$ CFU/ml.

Antimicrobial solutions were prepared and sterilised by filtering through a 0.2 μm

minisart single use sterile filter (Sartorius-Stedim Biotech, Fisherscientific, UK). Stock solutions

(1024 μg/ml) were prepared, taking into account the stated product purity. Reduction of

peptide concentration through filtration was not assayed for.

50 μl of each agent, at a stock concentration of 1024 μg/ml, was added to well 1 of a 96-

well microplate (Fisherscientific, UK) and a two-fold dilution series prepared in wells 2 to 12. 50

μl of inoculated TSB, containing $1x10^6$ CFU/ml of *A. baumannii*, was then added to each well

115 resulting in final peptide concentrations from 256 µg/ml to 0.125 µg/ml and a cell density of

116 5×10^5 CFU/ml. Plates were covered and incubated in a shaking incubator at 37 °C and 140 rpm

117 for 24 h. The lowest concentration of peptide where the well was visibly clear was recorded as

118 the MIC. The entire experiment was carried out three times in triplicate to give nine datasets

119 and the mean MIC established.

Minimum bactericidal concentration (MBC)

121   After recording the MIC, spread plates were prepared on TSA using 100 µl from each

122 clear well. These plates were incubated at 37 °C for 24 h. The lowest concentration where there

123 was no growth observed on the plate was recorded as the MBC. This was carried out after each

124 MIC test and therefore three times in triplicate.

Minimum biofilm inhibitory concentration (MBIC)

126   A 96-well microplate plate containing doubling dilutions of antimicrobial was incubated

127 with 5×10^5 CFU/ml *A. baumannii* for 24 h at 37 °C, 140 rpm. After 24 h, the wells were emptied

128 and washed three times with ¼ strength Ringer's solution and air dried for 1 h. 1% crystal violet

129 solution was added to each well and left at room temperature for 10 minutes. The wells were

130 emptied by pipetting, washed three times with distilled water, and air dried for 30 minutes at

131 37 °C. The stain was solubilised with 96% ethanol (100 µl). The plate was covered and shaken at

132 140 rpm for 30 minutes. 2 µl was removed and the absorbance of the solution was measured at

133 590 nm compared to a 96% ethanol blank. This was replicated three times and the mean

134 calculated. The percentage biofilm inhibited was calculated by comparing against an untreated

135 bacterial control. SPSS Statistics 21 (IBM, USA) was used for the statistical analysis of MBIC

136   results. The samples t-test was used to find if there was a statistical difference of 95% ($p \leq 0.05$,

137   statistically significant) or 99% ($p \leq 0.01$, highly significant).

Biofilm removal assay

139   Biofilms of *A. baumannii* were grown in 36 wells of a 96-well microplate (test plate) by

140   adding 50 µl sterile TSB and 50 µl of TSB inoculated with 1×10^6 CFU/ml culture. Plates were

141   incubated at 37 °C, 140 rpm for 24 h. On a separate 96-well microplate (titration plate) a

142   doubling dilution series was prepared with 75 µl of TSB and 75 µl of antimicrobial solution

143   (stock concentration, 8192 µg/ml). The wells containing biofilm were washed three times with

144   ¼ strength Ringer's solution and 75 µl sterile TSB was then added to each well. 75 µl from the

145   titration plate, containing a specific concentration of peptide, was added to the corresponding

146   well on the test plate. This resulted in each well containing 150 µl of TSB, which ensured the

147   biofilm was completely submerged, and the test wells containing a doubling dilution series of

148   antimicrobial (2048 µg/ml to 2 µg/ml). 150 µl sterile TSB was added to the negative control

149   wells. Plates were covered and incubated in a shaking incubator for 24 h at 37 °C, 140 rpm.

150   After incubation, the wells were emptied by tipping onto absorbent paper, washed three times

151   with 200 µl ¼ strength Ringer's solution and air fixed for 1 h under aseptic conditions.

152   The percentage of biofilm removal was quantified by measuring the absorbance after

153   applying crystal violet stain as described previously. The absorbance (at 590 nm) was

154   determined for each sample. Biofilm reduction (%) was determined by comparison of the

155   absorbance readings of the samples with the untreated control biofilms.

156   SPSS Statistics 21 software (IBM, USA) was used to assess the significance of the biofilm

157   removal assay results. The samples t-test was applied to assess the statistical difference of 95%

158   (p≤0.05) which was considered as statistically significant or 99% (p≤0.01) which was considered

159   highly significant.

160   **Scanning electron microscopy**

161   *A. baumannii* biofilms were prepared in 150 µl TSB in 96-well microplates. Calgary

162   Biofilm Device (CBD) lids (Nunc-TSP Screening Plate Lids, ThermoScientific, UK) were placed on

163   the plates with the polystyrene pegs protruding into the broth. Plates were sealed and

164   incubated at 37 °C, 140 rpm for 24 h. After incubation, the pegs were washed by submerging in

165   200 µl of sterile ¼ strength Ringer's solution and leaving for 2 minutes and this process was

166   repeated twice more. Each well was then filled with 100 µl sterile TSB.

167   Antimicrobial challenge plates were prepared, containing agent concentrations of 1

168   µg/ml, 10 µg/ml and 100 µg/ml. The CBD lid was placed onto this challenge plate, covered and

169   incubated at 37 °C, 140 rpm for 24 h. After 24 h the pegs were washed by submerging the CBD

170   lid in 250 µl of sterile ¼ strength Ringer's solution and leaving for 2 minutes and subsequently

171   repeated twice more.

172   0.1M sodium cacodylate buffer was prepared to give a pH reading of 7.2. The CBD pegs

173   were then submerged in this solution for 3 minutes.

174   2.5% glutaraldehyde solution was prepared. The CBD lid was immersed in the buffer,

175   covered and left at ambient temperature (21 ± 2 °C) for 40 minutes. The CBD lid was then

176   removed and twice washed in distilled water for 2 minutes. Biofilms were then dehydrated

177   sequentially by placing twice into 50% methanol (Fisher Scientific, UK), 70% methanol and

178   finally 100% methanol. CBD lids were left in each solution for 2 minutes. Pegs were

179   subsequently air dried for 4 days.

180   Pegs were removed from the CBD lid and mounted onto 0.5" aluminium specimen

181   stubs, fixed using carbon adhesive discs (Agar Scientific, UK). After mounting, the carbon tabs

182   were painted with graphite and the pegs were coated with approximately 15-20 nm of

183   platinum. *A. baumannii* biofilms on the surface of the pegs were visualised using a FEI Quanta

184   FEG 650 Scanning Electron Microscope.

185   **Atomic force microscopy**

186   Atomic force microscopy (AFM) was used to obtain topographic images of *A.*

187 *baumannii* cells when subjected to peptide concentrations equating to ½ MBC, MBC and 2x

188 MBC. These concentrations were 0.25, 0.5 and 1 µg/ml for colistin and 2, 4 and 8 µg/ml for both

189 bicarinalin and BP100. 150 µl suspensions, containing inoculated TSB with *A. baumannii* cell

190 density of 5×10^5 CFU/ml and appropriate concentrations of antimicrobial, were prepared in 96-

191 well microplates. Plates were incubated at 37 °C, 140 rpm for 2 h.

192   After incubation the suspension was pipetted onto a poly-L-lysine coated slide (Sigma

193 Aldrich, UK) and left at ambient room temperature (21 ± 2 °C) for 20 minutes. Slides were

194 rinsed with distilled water and left to air dry.

195   Images were captured using a Bioscope Catalyst AFM (Bruker, Germany) operated in

196 PeakForce tapping mode using ScanAsyst-air tips (Bruker, Germany). Images were acquired on

197 an area of 4 μm^2 at a scan rate of 0.5 Hz.

198

Vesicle leakage assays

Liposomes were made from lipids extracted from *A. baumannii*. *A. baumannii* cultured at 37 °C was harvested and a total lipid extraction conducted as described by Bligh & Dyer [27], with an extra step to extract any lipid remaining in the supernatant plus 3x washing steps with 1M KCl. The extracted lipid was stored in chloroform at -20 °C. A Stewart lipid assay was conducted to quantify the lipid extracted.

2 mg lipid, dried from chloroform in a round-bottomed flask, was hydrated in 1 ml 100 mM 5(6)-carboxyfluorescein (Sigma Aldrich, UK) and allowed to hydrate for 25 minutes with occasional shaking. The resulting suspension was extruded through a 400 nm filter and then a 100 nm Nuclepore track-etched polycarbonate membrane (Whatman, UK) using a mini-extruder (Avanti Polar Lipids, USA) at 37 °C. The extruded liposomes were then washed 3 times to remove un-encapsulated carboxyfluorescein by pelleting at 100,000 g and re-suspending in 50 mM Tris-HCl buffer and 10 mM NaCl at pH 7.3 before final re-suspension in 1 ml of the same buffer.

6.25 μl of the above liposome suspension was added to 500 μl aliquots of antimicrobial peptide solutions at the following concentrations: 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 & 512 μg/ml in the above Tris buffer. An additional concentration of 0.125 μg/ml was used for colistin due to its lower MIC. 100 μl aliquots were transferred to black 96-well microplates for fluorescence measurements at 490 nm excitation and 520 nm emission using an Infinite 200Pro (Tecan, Switzerland). Carboxyfluorescein leakage was measured relative to the same quantity of liposomes suspended in 1% SDS following approximately 30 minutes exposure to the agent.

Results

Antimicrobial assays

220

221

222   MIC and MBC values were determined to be 0.5 µg/ml (0.43 µmol/L) for colistin, 4

223 µg/ml (1.8 µmol/L) for bicarinalin and 4 µg/ml (2.8 µmol/L) for BP100 (data not shown). Fig. 1

224 shows that the MBIC was found to be similar to the MIC and MBC for each agent with 90% of

225 biofilm formation inhibited at 0.5 µg/ml colistin and 4 µg/ml bicarinalin and BP100. This also

226 showed that at concentrations below the peptides' respective MBCs, biofilm formation was

227 possible with only 36% biofilm inhibition for colistin, 23% for bicarinalin and 20% for BP100. Fig.

228 2 shows the eradication of a 24-hour biofilm when subjected to increasing concentrations of

229 peptide. At 1 µg/ml of colistin, 27% of biofilm was removed. As the peptide concentration

230 increased, more biofilm was eradicated with 52% removed at 512 µg/ml. Bicarinalin removed

231 18% at 1 µg/ml which then increased to 70% at 128 µg/ml; no further biofilm eradication was

232 observed with higher concentrations. BP100 removed 17% at 1 µg/ml which remained low until

233 16 µg/ml. Above this concentration, the biofilm removed increased from 27% at 32 µg/ml to

234 76% at 512 µg/ml, higher than both colistin and bicarinalin, to 93% at 2048 µg/ml.

Microscopy

235

236   SEM (Fig. 3) and AFM (Fig. 4) provide visualisation of the effects of these agents on *A.*

237 *baumannii* cells. Fig. 3 demonstrates that, as the concentration of the agents increases, the

238 morphological changes to the cells become more pronounced including evidence of blebbing

239 and a more variable cell shape with shrinkage and membrane disruption. The coverage across

240 the surface also reduces: with no addition of antimicrobial agent the cells are packed closely

241 together and cover the whole interface; as the peptide concentration increases visible gaps are

242   seen between clusters of cells. The cellular morphological changes are also observed in Fig. 4 by

243   AFM. Blebbing is observed, causing varied cell shape and shrinkage at the MBC concentrations,

244   and the cells differ greatly compared to the cocci-bacilli shaped cells seen with no antimicrobial

245   treatment and below the MBC.

246   **Vesicle leakage assays**

247   To determine the mechanism of action of the antimicrobial agents, a vesicle leakage

248   assay was performed. Liposomes produced from lipids extracted from *A. baumannii* (Fig. 5) all

249   leaked carboxyfluorescein in the presence of the antimicrobials. The concentration of agent

250   causing half-maximal leakage for each agent was ~ 1.75 µg/ml for colistin, ~ 2.75 µg/ml for

251   BP100 and ~ 2 µg/ml for bicarinalin (given maximal leakage of 70%, 65% and 56% respectively).

252   Vesicle leakage results support the proposal that these agents exert effects on the bacterial cell

253   membrane causing significant disruption to the cell surface (as shown in Fig. 4) and eventually

254   cell lysis (as shown in Fig. 3).

Discussion

A. baumannii susceptibility to colistin was similar to that determined by Li *et al*. [28] and Sauger

[29], confirming that *A. baumannii* 19606 strain is colistin susceptible as defined by The Clinical

and Laboratory Standards Institute. Bicarinalin results were compared with those of Rifflet [18]

and Téné [19] who investigated *Cronobacter* spp., *Enterobacter* spp. and *Staphylococcus* spp..

Results suggest that *A. baumannii* is generally more susceptible to bicarinalin than these other

bacteria. Antimicrobial activity results for BP100 against *A. baumannii* 19606 were comparable

to other bacteria in previous studies [20, 21].

Although the MBC for both bicarinalin and BP100 was 4 µg/ml, the molar concentration of

peptide differed (1.8 µmol/L for bicarinalin and 2.8 µmol/L for BP100). This is primarily due to

the differences in molecular weight (bicarinalin 2213.78 g/mol, BP100 1420.88 g/mol) indicating

that, although BP100 is smaller in length, it exerts a similar inhibitory and potent effect on *A.*

baumannii. By comparison, colistin has a MBC of 0.43 µmol/L (with the smallest molecular

weight, 1155.4 g/mol).

MBIC results demonstrated that, at the MBC for all three antimicrobials studied, there was less

than 10% biofilm formed compared to the control. Additionally, at concentrations of

antimicrobial agents equal to ½ MBC (2 µg/ml for bicarinalin and BP100, 0.25 µg/ml for colistin)

there was less inhibition of biofilm formation with 77% of biofilm formed in the presence of

bicarinalin, 95% in BP100 and 64% in colistin. This highlights that even below their respective

MBCs, bicarinalin and colistin have some potential to prevent biofilm formation but BP100 had

very little, possibly due to differences in size and mechanism of action.

276

277 Biofilm eradication results indicated that bicarinalin is superior at eradicating *A. baumannii*

278 biofilms than colistin above its MBC and both bicarinalin and BP100 are more effective at

279 concentrations above 128 μg/ml. Colistin was more effective at removing *A. baumannii* biofilms

280 at low concentrations; this was as expected due to its lower MBC which is nearly an order of

281 magnitude less than bicarinalin and BP100. However, the maximum biofilm removed was only

282 ~50% at 512 μg/ml. Bicarinalin was the most effective between 8 μg/ml, where it removed

283 ~40% of the biofilm, and 128 μg/ml, where it eradicated 70% of the biofilm. Any further

284 increase of concentration had no additional significant effect on the biofilm. The removal

285 activity of BP100 started slowly and at 16 μg/ml it had removed only 20% of the biofilm, half

286 that of colistin. Above 16 μg/ml, BP100 increased relatively linearly to exceed bicarinalin activity

287 above 512 μg/ml. At 2048 μg/ml, the highest concentration tested, BP100 had eradicated ~95%

288 of the biofilm. Colistin had the most biofilm removal activity, as expected, at low concentrations

289 of 1-4 μg/ml, below the MBC of bicarinalin and BP100. Bicarinalin was the most active between

290 8 and 128 μg/ml and BP100 was the most effective from 256 to 2048 μg/ml. The superior

291 performance of bicarinalin, and particularly that of BP100 on biofilms at higher concentrations,

292 was not anticipated especially considering the MBC is significantly higher than colistin.

293 The SEM has been widely used to image bacterial biofilms [30, 31], revealing, with increasing

294 concentrations of peptide, progressive changes in cellular shape and structure of the biofilm.

295 Membrane protrusions between bacterial cells were seen in the control sample and at 1 μg/ml

296 concentrations for each agent in this study. Pili or cellular filaments between cells have been

297 previously documented between cells within biofilms [32, 33]. At the higher concentrations of

298 10 µg/ml and 100 µg/ml, these protrusions were not visible suggesting the inability of the cells

299 to form pili at these concentrations [34]. Fig. 3 clearly shows that as the concentration of agent

300 is increased, the cell shape becomes less regular and the surface coverage is decreased.

301 The effect of the antimicrobials on *A. baumannii* was visualised by AFM. Fig. 4 highlights that,

302 below the peptide MBC, the coccus-bacillus cell shape is conserved. However, at concentrations

303 at and above the MBC, the cell surface, conformation and size are drastically affected. At the

304 highest concentrations tested there is evidence of severe disruption to the cell surface,

305 cytoplasmic leakage and lysed cells.

306 Membrane disruption caused by peptide activity is noted from various studies [20, 35-37]. In

307 this study, indentations and pores are seen in the bacterial surface with resulting cytoplasmic

308 leakage and debris, suggesting loss of turgor pressure [38]. As the peptide concentration

309 increased, more intense membrane perturbation and cytoplasmic leakage were visualised.

310 Similar observations were described by Li *et al*. [35].

311 Although AFM imaging of bacterial cells in air is a widely used technique [20, 39], it can lead to

312 dehydration of the cell and changes in the cell surface that were not directly caused by peptide

313 activity. However, all images have been compared to the control of untreated cells.

314 The dye leakage assay reflects the relative potency of these peptides against *A. baumannii*,

315 suggesting that the peptides mediate their effects through the formation of pores in the

316 membrane although it likely reflects planktonic growth more accurately than biofilm-like

317 structures. It cannot be directly concluded whether the peptides exert their effects on the

318 cytoplasmic or outer membrane of the cell. The visualised bacterial cell morphological changes

319   seen in SEM (Fig. 3) and AFM (Fig. 4) images are likely to relate to outer membrane disorder

320   while the vesicle leakage assay may represent disruption to the cytoplasmic membrane.

321   Overall the results support the proposal that bicarinalin and BP100 could be used to control

322   infections caused by biofilm-producing prokaryotes. However, Torcato *et al*. [40] observed 50%

323   cytotoxicity against HELA cells at 49.2±1.4 μmol/L for BP100 which is higher than the MBC

324   observed here (2.8 μmol/L) but lower than the concentrations needed for antibiofilm activity,

325   suggesting that clinical antibiofilm activity of BP100 may be unlikely. Colistin performs well

326   against planktonic cells of *A. baumannii* but significantly less well against biofilms. As *A.*

327   *baumannii* is able to form biofilms, it is essential that investigations into the efficacy of novel

328   agents are performed rigorously on biofilms.

329   The results indicate that bicarinalin and BP100 both have similar bactericidal and generally

330   better anti-biofilm activity against *A. baumannii* than colistin, with evidence supporting the idea

331   that these peptides mediate their effects through the formation of pores in the membrane. This

332   is encouraging but cytotoxic studies will determine whether, at these concentrations,

333   bicarinalin and BP100 can be used in clinical treatment.

334   **Conflict of interest**

335   There is no conflict of interest between any author and any other people or organisation that

336   could inappropriately influence this work.

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341   Science Research Ethics Committee, University of Lincoln, UK.

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438

Figure legends

439

440   Fig. 1: MBIC for colistin sulphate, bicarinalin and BP100. *A. baumannii* was subjected to a

441   doubling dilution series (2-2048 µg/ml) of antimicrobial for 24 h. After washing and air fixing for

442   1 h, samples were stained with 1% crystal violet. After 10 minutes the stain was solubilised with

443   96% ethanol and the absorbance at 590 nm was read against a 96% ethanol blank. The

444   percentage biofilm inhibited was calculated by comparing against an untreated bacterial

445   control. The experiment was carried out three times in triplicate.

446   Fig. 2: Percentage biofilm eradication by colistin sulphate, bicarinalin and BP100. *A.*

447   *baumannii* biofilms were grown for 24 h and then subjected to a doubling dilution series (2-

448   2048µg/ml) of antimicrobial for 24 h. After washing and air fixing for 1 h, samples were stained

449   with 1% crystal violet. After 10 minutes the stain was solubilised with 96% ethanol and the

450   absorbance at 590 nm was read against a 96% ethanol blank. The percentage biofilm eradicated

451   was calculated by comparing against an untreated bacterial control. The experiment was
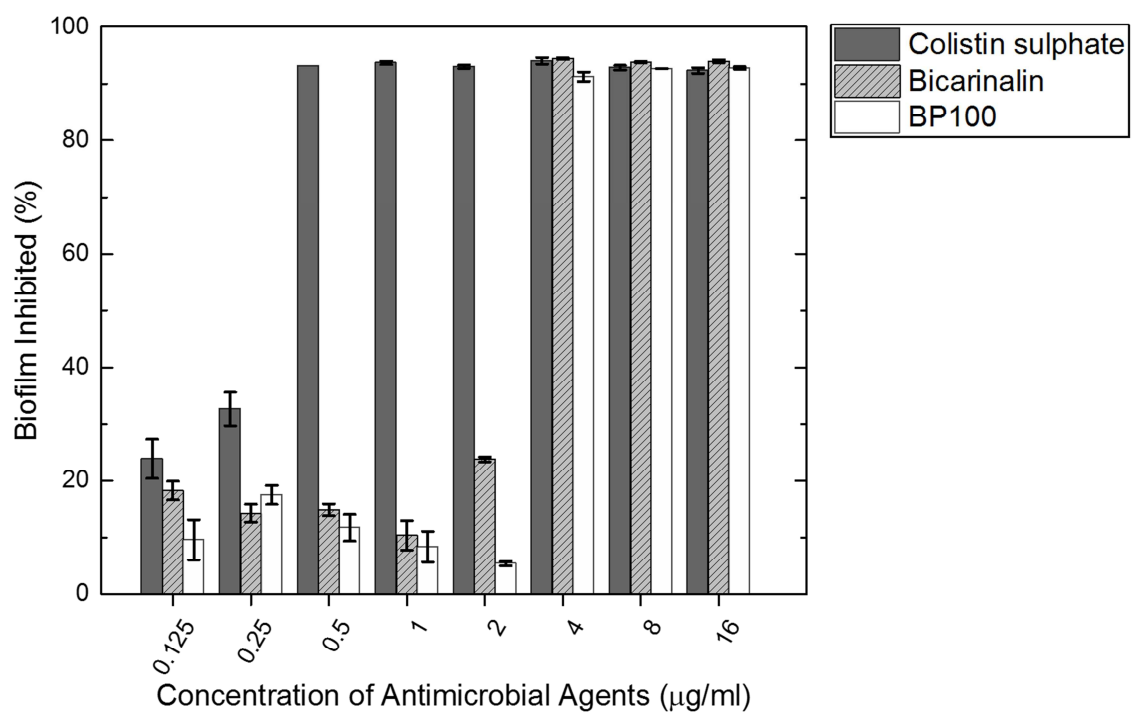
452   carried out twice in triplicate.

453   Fig. 3: SEM Images of *A. baumannii* biofilms. Biofilms were grown for 24 h then treated

454   for a further 24 h with colistin (A), bicarinalin (B) and BP100 (C). Control images, no

455   antimicrobial (1), 1 µg/ml (2), 10 µg/ml (3), 100 µg/ml (4). Scale bars on main images are 1 µm,
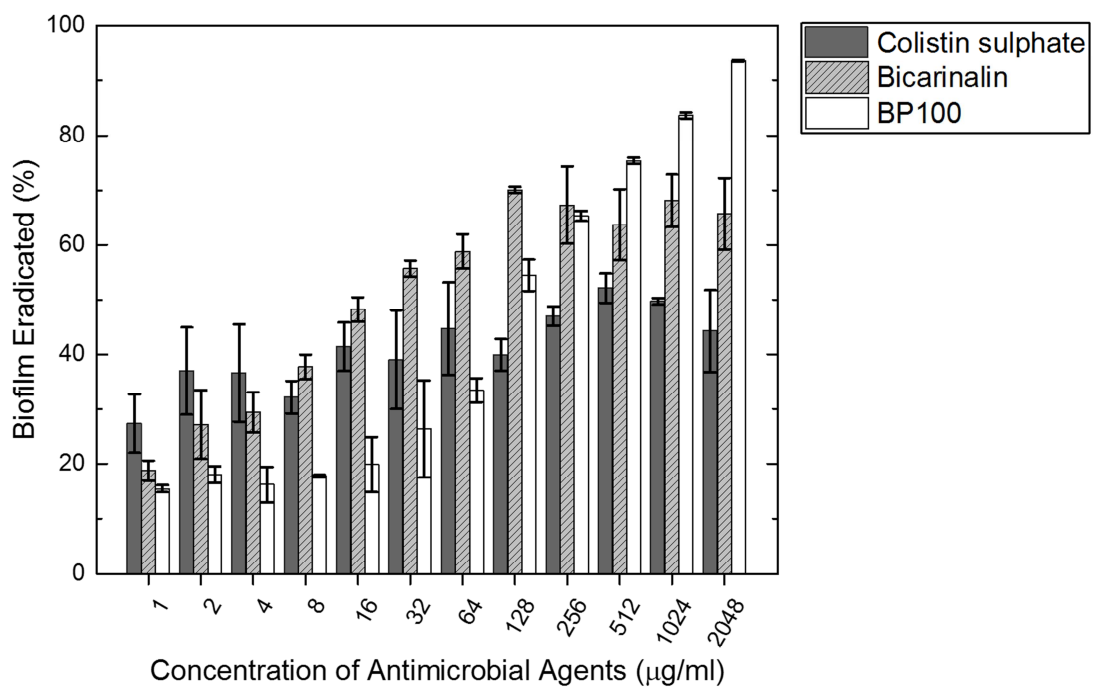
456   10 µm on inset images.

457   Fig. 4: AFM Images of *A. baumannii* cells after being treated with colistin (A), bicarinalin

458   (B) and BP100 (C) for 2 h and fixed onto a glass slide coated with poly-L-lysine. Control images,
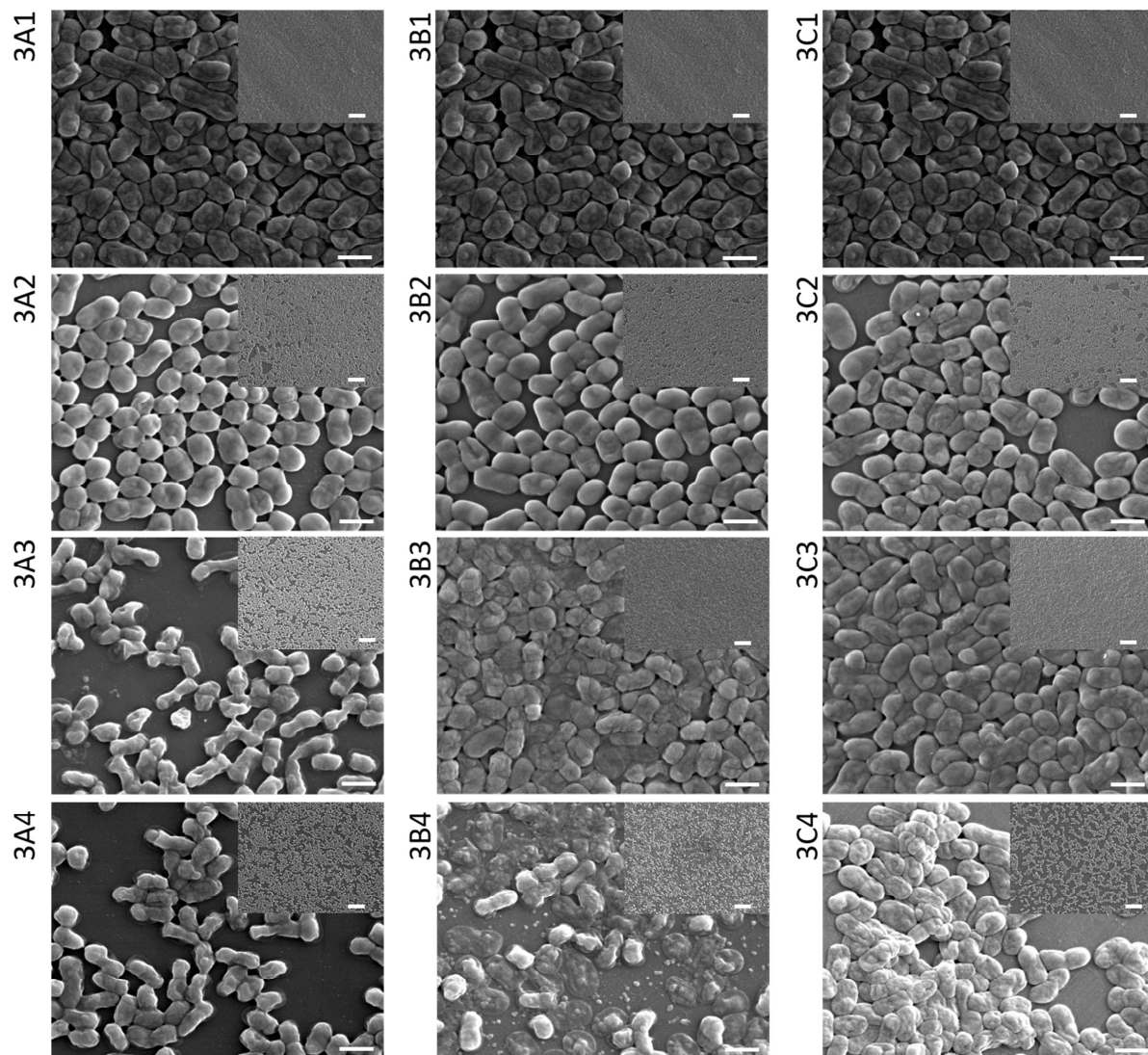
459   no antimicrobial (1), 1/2 MBC (2), MBC (3), 2X MBC (4). Images were 4 μm^2 and 256 lines at 0.5

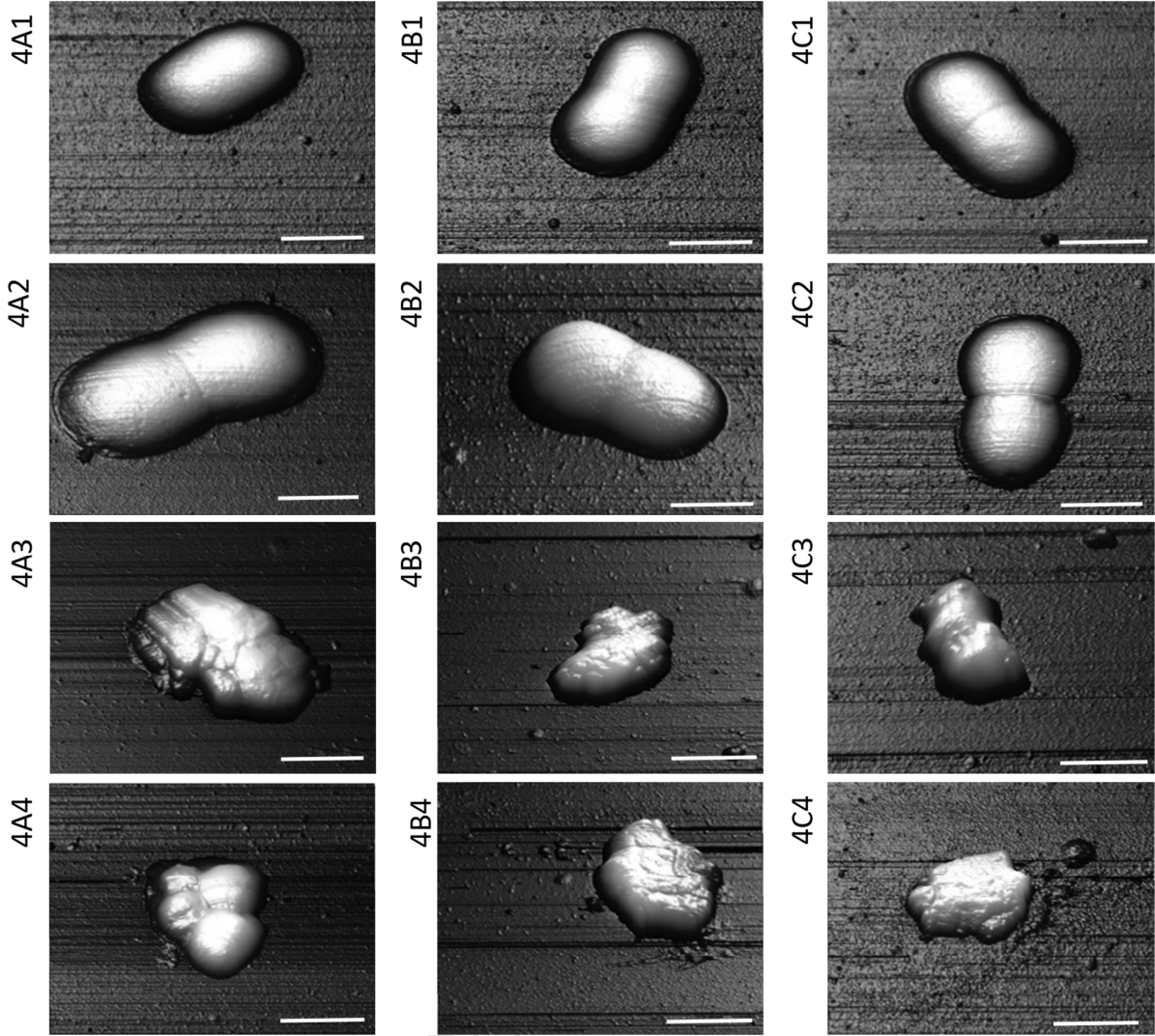460   Hz. Scale bars are 1 μm.

461   Fig. 5: The relative leakage of carboxyfluorescein from liposomes made from *A.*

462   *baumannii* total lipid extract in the presence of increasing concentrations of the three

463   antimicrobial agents. Error bars show the standard error around the mean of 3 replicates.

464   N.B. As instructed, panels of Figs. 3 & 4 have been provided on a single page. They can

465   be provided in separate files if required at a later stage.

4A1

4B1

4C1

4A2

4B2

4C2

4A3

4B3

4C3

4A4

4B4

4C4