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1 **Evaluation of a library of FDA-approved drugs for their ability to potentiate antibiotics against**  
2 **multidrug resistant Gram-negative pathogens**

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15 **Abstract**

16 The Prestwick library was screened for antibacterial activity or ‘antibiotic-resistance breaking’ (ARB)  
17 potential against four species of Gram-negative pathogens. Discounting known antibacterials, the  
18 screen identified very few ARB hits, which were strain/drug specific. These ARB hits included  
19 antimetabolites (zidovudine, floxuridine, didanosine, gemcitabine), anthracyclines (daunorubicin,  
20 mitoxantrone, epirubicin) and psychoactive drugs (gabapentin, fluspirilene, oxethazaine). This  
21 suggests that there are few approved drugs which could be directly repositioned as adjunct-  
22 antibacterials and these will need robust testing to validate efficacy.

23 **Main text**

24 The need for new antibiotics is driven by the rapid spread of multidrug resistant (MDR) bacterial  
25 pathogens and the absence of new antibiotics in the clinical development pathway is significant  
26 cause for concern. The idea of repurposing existing drugs, which are currently used as treatments for  
27 other disease areas is attractive because, due to the known safety profile of approved drugs, the  
28 cost and time to clinic could be significantly lower than novel scaffolds <sup>1</sup>. Examples of successful  
29 repurposing screens, outside of the antibacterial area, have produced candidates for Ebola, Zika  
30 virus and anti-cancer therapies <sup>2-4</sup>. Recent studies for the identification of new antibacterial leads  
31 have focussed on two key areas; i) identification of direct antibacterial hits for one or more target  
32 bacteria <sup>5,6</sup>, and ii) screening for compounds which synergise with existing antibiotics, thereby  
33 restoring activity of the antibiotic against strains/species which are currently resistant to their use <sup>7</sup>.  
34 Several previous studies identified antibacterial activities that are too weak to be effective on their  
35 own and would require exposures greater than the maximum concentration achievable with their  
36 primary pharmacology and recommended safe dosing <sup>7</sup>, possibly because of the bacterial membrane  
37 barriers.

38  
39 The current study aimed to identify either direct-acting antibiotics, or compounds which sensitise  
40 resistant Gram-negative strains to one or more antibiotics, looking to identify 'Antibiotic Resistance  
41 Breakers' (ARBs).

42 A high-throughput combination screen (HTCS) of potential ARBs and antibiotics was performed in  
43 384-well format from the Prestwick library of 1280 selected compounds in combination with five  
44 antibiotics or 0.1 % DMSO, in duplicate. Each replicate was from independent dilution plates by  
45 using independent inocula on two different days. The potential ARBs were tested at two  
46 concentrations, 20  $\mu$ M and 7  $\mu$ M, in combination with antibiotics at 0.125 x MIC. Concentrations  
47 were selected to balance the probability of achieving a significant number of hits with realistic  
48 concentrations which align with the likely C<sub>max</sub> for a typical drug. Where the MIC was >128 mg/L,  
49 the antibiotic was tested at 16 mg/L. The MICs of test articles were determined in cation-adjusted

50 Mueller-Hinton broth (caMHB; Oxoid), using the Clinical and Laboratory Standards Institute (CLSI)  
51 guidelines M7-A10 & M100-S26.

52 Clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and  
53 *Acinetobacter baumannii* which were recently highlighted by the World Health Organisation as  
54 priority pathogens for which new antibiotics are urgently required<sup>8</sup>, were selected which were  
55 resistant to each antibiotic. In some species (*K. pneumoniae* and *A. baumannii*), this involved use of  
56 two strains to cover all resistance profiles, and some resistance profiles were not available (Table  
57 S1).

58 During the HTCS, bacterial growth was determined by reading on a modal reader (Infinite 500,  
59 Tecan) at 600 nm after 24 h of incubation. For each plate, OD600 measurement was done at 2  
60 timepoints, T0 h (to determine the background signal related to the coloured compounds) and T24 h  
61 at the end of incubation. After blank substitution, calculated by subtracting OD600 at T0 h from  
62 OD600 at T24 h, a normalization step was carried out between OD600 values obtained in wells  
63 containing the compounds compared to those obtained in control wells (DMSO wells – maximal  
64 growth). Data analysis for each run was performed with Genedata Screener software. The workflow  
65 from the raw data associated to plate-map up to the normalization step was fully automated  
66 allowing for complete tracking of all data. The Z' factor and assay window were determined for each  
67 plate, between the positive control in presence of antibiotic at 0.125 x MIC and the negative control  
68<sup>9</sup>. The Z' factor for each combination of strain and antibiotic was between 0.5 – 0.8, plates displaying  
69 a Z' factor < 0.5 were automatically retested.

70 After statistical analysis, hits were defined as data points with an activity > hit threshold based on  
71 the Sigma method (mean + 3 standard deviations), unless otherwise stated. Results were expressed  
72 as percentage of growth inhibition compared to that in untreated controls (exposed to 0.1% DMSO  
73 only), assessed by optical density.

74 Firstly, compounds from the library were tested for direct antimicrobial activity at two  
75 concentrations, 7  $\mu\text{M}$  and 20  $\mu\text{M}$ , in the presence of 0.1 % DMSO (Figure S1 and S2). The number of  
76 direct hits at either concentration varied considerably between species, with 29 for *E. coli*, 16 for *P.*  
77 *aeruginosa*, 85 for the two *A. baumannii* strains combined and 53 for the two *K. pneumoniae* strains  
78 (discounting overlapping hits between the two strains of the same species and between the two  
79 concentrations tested) (Table S2). As might be expected we saw three scenarios with respect to dose  
80 response, i) compounds which were equally effective at both concentrations, ii) compounds which  
81 were effective at 20  $\mu\text{M}$  which were not effective as either direct antibacterials or ARBs at 7  $\mu\text{M}$  and  
82 iii) compounds which were ARBs at 7  $\mu\text{M}$  but which were directly antibacterial at 20  $\mu\text{M}$ .

83 Compounds at 7  $\mu\text{M}$  or 20  $\mu\text{M}$  were also tested in combination with antibiotics at concentrations of  
84 0.125 x MIC. There were few hits which overlapped between species (Figure 1). Most of the  
85 compounds which did overlap were known antimicrobials or antiseptics (Tables S5-S10). A number  
86 of compounds showed interesting potentiation, and these are discussed further below and in the  
87 supplementary file.

88 Three anthracycline-related molecules, daunorubicin, mitoxantrone and epirubicin showed  
89 potentiation with one or more combination of drug and species (Table 1). The pattern of activity  
90 differed between the three molecules tested, with no evidence of direct antibacterial activity, but  
91 differing levels of potentiation for other antibiotics.

92 Several nucleotide/nucleoside analogues, identified as antimetabolites and/or antiviral agents, also  
93 showed potentiation with one or more antibiotic (Table 1). Whilst simplistically such molecules  
94 might be expected to have similar effects, via interference with DNA/RNA metabolism in the cell,  
95 there were clear differences in the spectrum of activity between the compounds.

96 Two psychoactive compounds, fluspirilene and oxethazaine were also found to act as ARBs with  
97 colistin and merited further investigation, given the possibility that their mode of action might be

98 different to cationic compounds identified previously as able to potentiate colistin (for example  
99 pentamidine <sup>10</sup>, which was not found to potentiate colistin activity in this study, and cysteamine,  
100 which was not included in this study <sup>11</sup>). The MIC of colistin alone, and in combination with set  
101 concentrations of fluspirilene and oxethazaine was determined as above, but in non-cation adjusted  
102 Mueller-Hinton broth (Oxoid) and polypropylene plates, incubated for 20 hours at 37°C <sup>12</sup>.

103 Colistin potentiation by fluspirilene and oxethazaine in a wider panel of colistin-resistant strains of *K.*  
104 *pneumoniae* and a smaller number of other Gram-negative pathogens was tested as examples of  
105 compounds which were clear ARBs with very little direct antimicrobial activity (Table S3). The studies  
106 were designed as a fixed concentration synergy experiment, looking for ARB activity. Initially, MICs  
107 and growth curves were used to analyse direct effects of the two compounds. In most cases the MIC  
108 was >160 µM for *Klebsiella* spp. and *P. aeruginosa* isolates. For *E. coli*, all strains had an MIC of 160  
109 µM or above for oxethazaine, but two strains (LEC001 and 319238/UR) had MICs of 80 µM for  
110 fluspirilene. The notable exception to the high MIC values identified, were the *A. baumannii* strains,  
111 which showed an MIC of 20 µM for both oxethazaine and fluspirilene in both colistin-resistant  
112 strains (Table S4).

113 Despite being ARB hits with the original colistin-resistant *K. pneumoniae* strain used in the HTCS,  
114 within the broader panel of *Klebsiella* isolates, there were few examples of clear colistin potentiation  
115 with either compound. Only strains NCTC 13439 CST 2A (4-fold), MGH 78578 CST A (8-fold) and  
116 m109 CST 1B (32-fold) showed greater than 2-fold potentiation of colistin with fluspirilene (Figure 2,  
117 Table S3) and no strains showed this level of potentiation with oxethazaine.

118 In contrast, fluspirilene showed potentiation of colistin in all of the other Gram-negative species  
119 tested, with levels ranging from 4-fold (*A. baumannii* W1 CST\_R) to >128 fold (*E. coli* LEC001). The  
120 latter strain was also the only strain which showed potentiation with oxethazaine, again with  
121 increased susceptibility to colistin of >128 fold. Whether derivatives of fluspirilene merit further  
122 investigation as a stand-alone antibiotic or as an ARB, may depend on the novelty of its mechanism

123 of action. The developability is hampered by the relatively high concentration required to achieve  
124 potentiation of colistin, for example, around 20  $\mu$ M against *K. pneumoniae* (equivalent to 9.5 mg/L)  
125 compared to the daily dose (10 mg i.m. per day).

126 The current screen, in line with many other studies, suggests that there might be very few licensed  
127 drug compounds which could be simply repositioned, and which would have immediate benefit as  
128 adjunct therapies. This does not preclude future studies, looking at other antimicrobial strategies,  
129 such as, biofilm disruption <sup>5</sup>, anti-virulence compounds <sup>13</sup> or efflux pump inhibition <sup>14</sup>, but it does  
130 suggest that such studies must be carefully designed to generate useful information. The screening  
131 of existing approved drugs, while attractive from a regulatory standpoint and rapid route to market,  
132 does not directly address challenges of antimicrobial drug development, including the permeability  
133 issue which impacts on drug uptake into Gram-negative bacteria <sup>15</sup>, nor the relatively limited  
134 chemical space inhabited by most classical drugs <sup>16</sup>.

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200 interpretation.

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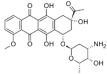
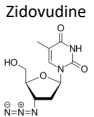

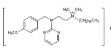
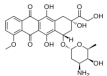
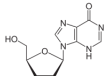

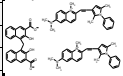
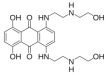
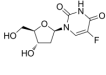
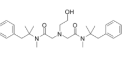
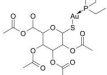
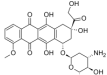
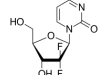
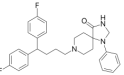
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215 **Table 1: Structures and antimicrobial profiles of interesting hits from the screen.** Shaded boxes  
216 illustrate direct or ARB activities, in  $\mu\text{M}$ , of compounds in combination with meropenem (MEM),  
217 ciprofloxacin (CIP), gentamicin (GEN), tigecycline (TGC) or colistin (CST) in the four Gram-negative  
218 species tested. Where compounds had activity at both 20  $\mu\text{M}$  and 7  $\mu\text{M}$ , only 7  $\mu\text{M}$  is represented in  
219 the table.

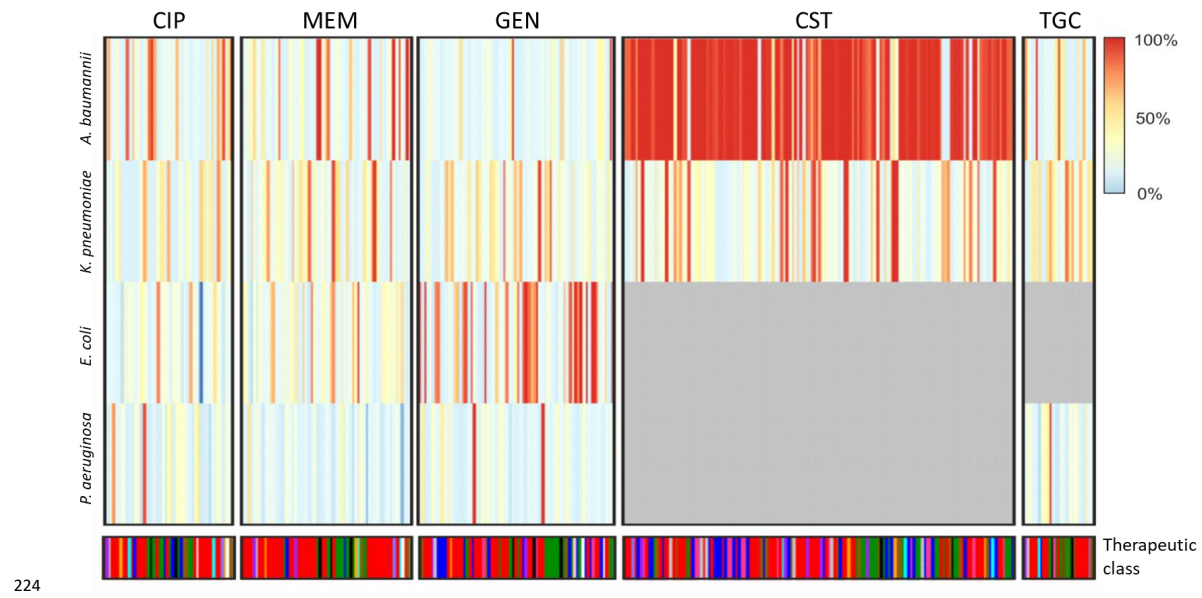


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Anthracyclines					Antimetabolites					Psychoactive					Miscellaneous													
		<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>			<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>			<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>			<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>					
 Daunorubicin	Direct					 Zidovudine	Direct	20	7			 Gabapentin	Direct					 Thonzonium bromide	Direct									
	MEM						MEM	7					MEM	20						MEM								
	CIP						CIP						CIP	20						CIP								
	GEN						GEN	7					GEN							GEN								
	TGC						TGC						TGC							TGC								
	CST	20	20				CST	7	7				CST							CST	7	7						
 Doxorubicin	Direct					 Didanosine	Direct	20				 Thiordazine	Direct					 Pyrinium pamoate	Direct									
	MEM						MEM	7					MEM						MEM					7				
	CIP						CIP						CIP						CIP									
	GEN						GEN						GEN						GEN						7	20		
	TGC						TGC						TGC						TGC									
	CST						CST	20	20				CST	20					CST	7								
 Mitoxantrone	Direct					 Floxuridine	Direct	20	7			 Oxethazaine	Direct					 Auranofin	Direct	7	20			7				
	MEM						MEM	7					MEM	20					MEM	7	20							
	CIP						CIP						CIP	20					CIP		20	20						
	GEN						GEN						GEN	20					GEN		7							
	TGC				20		TGC						TGC	20					TGC									
	CST						CST	20	7				CST	7	20				CST	7								
 Epirubicin	Direct					 Gemcitabine	Direct					 Fluspirilene	Direct	20														
	MEM						MEM						MEM															
	CIP						CIP			20			CIP															
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	TGC						TGC						TGC															
	CST	20	20				CST						CST	7	20													

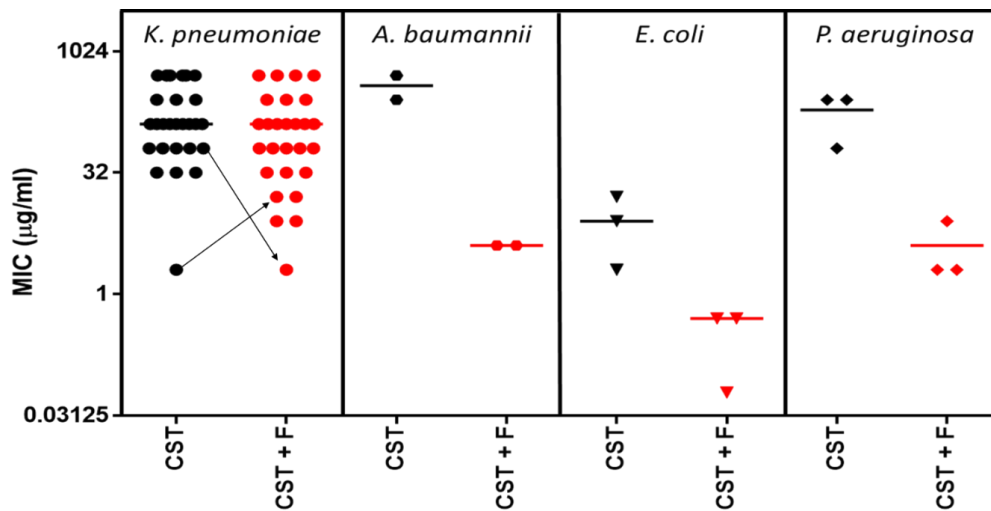
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221 **Figure 1: Few ARB hits show any conservation cross-species or with specific antibiotics.** Heat map showing ARB hits by species and antibiotic potentiated,  
 222 coloured according to the amount of growth inhibition they caused in each species in combination with each antibiotic. (grey is where the combination was  
 223 not tested).



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226 **Figure 2: Colistin ARB potential of fluspirilene.** A wider panel of colistin-resistant strains were tested  
 227 in the presence of fluspirilene. Although the *K. pneumoniae* strain used in the HTCS showed colistin-  
 228 potentiation by fluspirilene, this was not reflected in a wider panel. However, fluspirilene did  
 229 potentiate colistin in other Gram-negative species. Arrows on the *K. pneumoniae* panel indicate the  
 230 change in MIC for two specific strains. This highlights an example where fluspirilene is antagonistic to  
 231 colistin but where the MIC is in the same range as some strains where potentiation is observed.



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