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1       **Polyamino-isoprenyl derivatives as antibiotic adjuvants and motility inhibitors for**  
2               ***Bordetella bronchiseptica* porcine pulmonary infection treatment**

3  
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11

12    Keywords: Antibiotic resistance, Combination therapy, Whole-cell screening, motility,  
13    virulence, *Bordetella bronchiseptica*.

14 **Abstract**

15

16 The spreading of multidrug resistant bacteria and the lack of novel antibiotic molecules are  
17 leaving clinicians and veterinarians with very limited options to treat bacterial infections,  
18 especially those caused by Gram-negative pathogens. To reduce the selection of antibiotic  
19 resistance mechanisms and their transfer to human pathogens, veterinary pharmaceutical  
20 companies have dramatically decreased the number of used antibiotics. Among all the  
21 investigated alternate solutions, chemosensitizers, which decrease the amount of the used  
22 drugs appears to be one of the most promising strategy. In this study, we reported that  
23 polyamino-isoprenyl derivatives can potentiate florfenicol activity against veterinary sensitive  
24 reference strains as well as clinical isolates. These molecules induce inner membrane  
25 depolarization and subsequently inhibit efflux pumps by collapsing the proton motive force  
26 (PMF). Considering that *B. bronchiseptica* rotor flagellum is highly PMF dependent and that  
27 flagellar motility represents an important factor involved in colonization, we monitored the  
28 swimming and swarming motilities of bacteria and showed a strong inhibition in the presence  
29 of the lead selected compound. Taken together, our results suggest that this class of molecules  
30 are able to increase treatment efficacy and decrease drug consumption.

## 31 **Introduction**

32 In Europe, the overall sales of veterinary antimicrobial agents used in food producing  
33 animals represented 7680 tons in 2016  
34 (<https://bi.ema.europa.eu/analyticsSOAP/saw.dll?PortalPages>), and the main use (around  
35 60%) concerns the pig industry (Schwarz *et al.* 2001; Chauvin *et al.* 2002). The release of  
36 antibiotics in the environment by livestock production is one of the major contributing factors  
37 for the emergence of bacterial resistance in pathogens affecting both humans and animals  
38 (Jechalke *et al.* 2014, Wichmann *et al.* 2014, Bártíková *et al.* 2016, Albero *et al.* 2018). Thus,  
39 it is crucial to decrease the antibiotic consumption in veterinary field and particularly in  
40 livestock without affecting animal health (Wellington *et al.* 2014, Pruden *et al.* 2014).  
41 Consequently, strategies have been proposed, and one of the most successful consists of a  
42 dual drug approach allowing an efficient enhancement of antibacterial activity while  
43 minimizing the drug concentration (Brooks and Brooks 2014, Collignon *et al.* 2016).

44 The World Health Organization and the European Commission have published  
45 guidelines concerning the prudent use, in food production animals, of cephalosporin of 3<sup>rd</sup> and  
46 4<sup>th</sup> generation and fluoroquinolones, which are considered as “critically important”  
47 antimicrobial agents in human medicine  
48 (<https://bi.ema.europa.eu/analyticsSOAP/saw.dll?PortalPages>, Briyne *et al.* 2014). According  
49 to the international recommendations, the antimicrobials that could be used to treat porcine  
50 respiratory tract infections are tetracyclines, phenicols, macrolides, fluoroquinolones or a  
51 combination of trimethoprim and sulfonamides (Kadlec *et al.* 2004, Kadlec *et al.* 2007). It is  
52 noteworthy that respiratory diseases in swine such as pneumonia and atrophic rhinitis are  
53 caused by the Gram-negative coccobacillus *Bordetella bronchiseptica* that requires a  
54 tetracycline treatment even if resistance can occur (Prüller *et al.* 2015, Kadlec *et al.* 2018).  
55 Otherwise, florfenicol is a good alternative (Chauvin *et al.* 2002), because this antibiotic has a  
56 good distribution in the body and few side-effects. However, although florfenicol was only  
57 used in veterinary medicine, a cross-resistance by efflux with its non-fluorinated analogue,  
58 chloramphenicol has been evidenced (Kadlec *et al.* 2007).

59 In an ongoing project dedicated to reducing the florfenicol consumption from breeding  
60 pig holdings, we screened polyamino-isoprenyl compounds that were previously described  
61 (Borselli *et al.* 2016) for their ability to potentiate florfenicol activity. The objective of this  
62 study is to identify a compound with significant activity and to elucidate its mechanism of  
63 action on the membrane physiology and to evaluate its impact on the bacterial motility.

## 64 **Materials and methods**

65

### 66 **Bacterial strains and growth conditions**

67 The *B. bronchiseptica* strain used in this study comes from the Institut Pasteur collection, CIP  
68 55.110. Ten isolates from pig husbandries in France were obtained from Labofarm (Loudéac  
69 France) (Table 1). The bacterial identification was carried out by matrix-assisted laser  
70 desorption ionization-time of flight (MALDI-TOF) Biotyper (Bruker, Billerica, Germany)  
71 with comparison with the manufacturer's bacterial database. All the strains were grown either  
72 on Columbia agar at 35°C for 24h to 48h or in Columbia broth at 35°C for 24h.

73

### 74 **Antibiotics and Chemicals**

75 The antibiotic florfenicol was supplied by the pharmaceutical company Virbac (Carros,  
76 France). The antibiotic imipenem was purchased from Sequoia Research Products  
77 (Pangbourne, UK). All the chemicals Polymyxin B, Polymyxin B nonapeptide (PMBN),  
78 Phenylalanine-arginine beta-naphthylamide (PABN), Carbonylcyanide m-  
79 chlorophenylhydrazone (CCCP), Cetyl trimethylammonium bromide (CTAB), Dimethyl  
80 sulfoxide (DMSO), 3,3'-Dipropylthiacarbocyanine iodide (DiSC<sub>3</sub>(3)), Hydroxyethyl  
81 piperazineethanesulfonic acid (HEPES), Ethylene diamine tetraacetic acid (EDTA) and  
82 Benzalkonium chloride were purchased from Sigma Aldrich (St Quentin Fallavier, France).  
83 The polyamino-isoprenyl derivatives chemolibrary was described previously (Bolla *et al.*  
84 2012).

85

### 86 **MIC determination**

87 Minimal inhibitory concentrations of antibiotics (MIC) were measured by the microdilution  
88 broth method in 96-well microtiter plates using the two-fold dilution method and according to  
89 the NCCLS guidelines. The inoculum was adjusted at 5x10<sup>5</sup> CFU/mL in a final volume of  
90 200 µL. MIC was visually determined after 24 hours incubation at 37°C. Assays were  
91 performed in three independent biological experiments.

92

### 93 **High content screening**

94 The polyamino-isoprenyl derivatives chemical library was screened at 10 µM final  
95 concentration in the absence and in the presence of sub-inhibitory concentration of florfenicol  
96 (MIC/4) to identify only antibiotic adjuvants. We also added control molecules as polymyxin  
97 B, PABN, CCCP, CTAB and benzalkonium chloride at 10 µM. The screening was performed  
98 in 96-well microtiter plates in Columbia broth with a final volume of 200 µL and a DMSO  
99 final concentration of 2.5%. The bacterial inoculum was adjusted to 5x10<sup>5</sup> CFU/mL.  
100 Microtiter plates were read at 600 nm on an Infinite M200 Pro plate reader (Tecan, Lyon,  
101 France) after 24h incubation at 35°C. Assays were performed in three independent biological  
102 experiments.

103

### 104 **General procedure for the synthesis of compounds for the hit to lead procedure**

105 The general synthetic pathway is illustrated for the preparation of compound 1.

106 A mixture of farnesal (345 mg, 2.27 mmol), titanium(IV)isopropoxide (645 mg, 2.27 mmol)  
107 and spermine (2.27 mmol) in absolute methanol (5 mL) was stirred at room temperature for  
108 12 hours. Sodium borohydride (172 mg, 4.5 mmol) was then added at 0°C and the resulting  
109 mixture was stirred for an additional 2 hours. The reaction was then quenched by adding  
110 water (1 mL). Stirring was maintained at room temperature for 20 minutes. After filtration  
111 over a pad of Celite washing with methanol and ethylacetate, the solvents were removed  
112 under vacuum and the crude amine was purified by flash chromatography on silicagel. using

113 CH<sub>2</sub>Cl<sub>2</sub>/MeOH/ NH<sub>4</sub>OH (7/3/1) as eluent affording the expected coupling product 1 in 64%  
114 yield (see also supplementary material).

115  
116

### 117 **Checkerboard assay**

118 Combinations of polyamino-isoprenyl derivatives with florfenicol were tested by using the  
119 previously described microdilution checkerboard method (Kadlec *et al.* 2018). Briefly, an  
120 inoculum (100  $\mu$ L) of  $5 \times 10^5$  CFU/mL of *B. bronchiseptica* strain was added to 96-well  
121 microtiter plates containing a serial two-fold dilution of antimicrobial agents in Columbia  
122 broth. After incubation of plates during 24 h at 35°C, we determined the MIC for each  
123 combination and calculated the fractional inhibitory concentration indexes (FICIs) allowing  
124 us to characterize the level of efficiency of the combination. The FICIs are calculated as  
125 follows:  $FICI = FIC A + FIC B$ , where  $FIC A = MIC \text{ antibacterial A in combination} / MIC$   
126  $\text{antibacterial A alone}$  and  $FIC B = MIC \text{ antibacterial B in combination} / MIC \text{ antibacterial B}$   
127  $\text{alone}$ . The antibacterial combination was considered synergistic when the FICI was  $\leq 0.5$ ,  
128 indifferent when  $0.5 < FICI \leq 4$  and antagonistic when  $FICI > 4$  (Odds 2003).

129

### 130 **Outer membrane permeabilization assay**

131 100 milliliters of Columbia broth were inoculated with an overnight culture of a *B.*  
132 *bronchiseptica* isolate producing  $\beta$ -lactamases (isolate 77-A12). Once the cultures reached an  
133 the mid-logarithmic phase, imipenem was added (0.125 mg/L final concentration) for 2 hours,  
134 then cells were recovered by centrifugation (3,600 X g, 20 min, 20°C) and washed twice in 20  
135 mM potassium phosphate buffer (pH 7.2) 1 mM MgCl<sub>2</sub> (PPB) supplemented with 30  $\mu$ M of  
136 CCCP that allows the inactivation of active efflux (Nagano and Nikaido 2009). After the  
137 second centrifugation, the cell suspension was adjusted to 0.5 OD 600 nm. One hundred  
138 microliters of the bacterial suspension were mixed with 50  $\mu$ L of compound derivatives  
139 dilutions already set up in microplates, to give final concentrations ranging from 1.56 to 200  
140  $\mu$ M. Then, 50  $\mu$ L of nitrocefin was added to obtain a final concentration of 50  $\mu$ g/mL. The  
141 absorbance at 490 nm was monitored to follow the nitrocefin hydrolysis using a Sunrise  
142 microplate reader (Tecan) over 2 hours and 30 minutes. Experiments were performed in  
143 triplicate. For each compound, the efficacy of permeation was determined using the slope in  
144 the linear range, relatively to the control slope obtained with 100  $\mu$ M polymyxin-B.

145

### 146 **Real Time efflux assay**

147 The strain CIP55.110 was inoculated into 20 mL of Columbia broth and grown for 24 h at  
148 35°C to reach the stationary phase. Cells were recovered by centrifugation (3,600 X g 20 min,  
149 20°C) and washed twice in PPB. The cell suspension was adjusted to 0.5 OD 600 nm and  
150 loaded with 1,2' dinaphthylamine (TCI-Europe SA, Zwinjndretch, Belgium) 32  $\mu$ M final  
151 concentration, supplemented with the proton dissipator CCCP 30  $\mu$ M. After overnight  
152 incubation, cells were washed in PPB and the bacterial suspension was adjusted to 0.5 OD  
153 600 nm. In a 96-well Greiner black microplate (Greiner, Courtabœuf, France) 50  $\mu$ L of the  
154 desired tested compounds were mixed with 100  $\mu$ L of the cell suspension yielding final  
155 concentration ranging from 12.5 to 200  $\mu$ M. The dye transport was triggered after addition of  
156 5  $\mu$ L of Columbia broth and the fluorescence was monitored each 22 second during 30 min on  
157 an Infinite M200 microplate reader (Tecan) (excitation wavelength 370 nm and emission  
158 wavelength 420 nm). Polymyxin B is used as control in our experiments, this antibiotic is able  
159 to rapidly disrupt the proton-motive force without interfering with fluorescence  
160 measurements. Assays were performed in three independent biological experiments.

161

### 162 **Inner Membrane depolarization assay**

163 An overnight culture of bacterial strain CIP 55.110 was diluted 50-fold into 20 mL Columbia  
164 broth. After reaching an OD 600 nm of 0.5, cells were recovered by centrifugation (3,600 X  
165 g, 20 min, 20°C) and incubated 5 min at room temperature in 5 mM HEPES-10 mM EDTA  
166 (pH 7.0). They were then centrifuged (3,600 X g for 20 min at 20°C) and re-suspended in 5  
167 mM HEPES (pH 7.0) supplemented with Columbia Broth 2.5% and adjusted to an OD 600  
168 nm of 0.29. Fluorescence was monitored every 30 seconds (excitation wavelength 622 nm,  
169 emission wavelength 690 nm) after addition of 5 µL of a 160 µM solution of DiSC<sub>3</sub>(3) to 100  
170 µL of the cell suspension to reach a final concentration of 7.6 µM. After a period of  
171 incubation (16.5 min) to allow the dye incorporation into the polarized membranes, 10 µL of  
172 compound dilutions were added. The difference in the relative fluorescence values (RFU)  
173 from the control containing only buffer and the control containing untreated bacteria in buffer  
174 is taken as the maximum level of depolarization. Assays were performed in three independent  
175 experiments.

176

### 177 **Motility assay**

178 Swimming motility was evaluated on tryptone-agar plates (tryptone (1% w/v), 0.5 % NaCl  
179 (w/v), yeast extract 0.5 % (w/v)) supplemented with agar 0.3 % (w/v) (Murray *et al.* 2006).  
180 Swarming motility was evaluated on tryptone-agar plates supplemented with agar 0.5 % (w/v)  
181 (Murray *et al.* 2006). For both assays, sterilized tryptone-agar was dispensed to Petri dishes  
182 containing either no compound or compound **1** (Cpd**1**) at 10 µM and left to dry at room  
183 temperature for 2h. An inoculum obtained after dilution of an overnight culture was adjusted  
184 to an OD 600 nm of 1.1. The plates were inoculated with a sterile toothpick and incubated in a  
185 non-reverse position at 35°C during the indicated times (24h, 48h or 72h) and halo diameters  
186 corresponding to bacterial growth were measured and reported. Assays were performed in  
187 three independent biological experiments.

188

### 189 **Microscopy live tracking and acquisition**

190 A drop of each bacterial culture in exponential growth was deposited on a microscope slide  
191 and Cpd**1** was added where indicated and immediately observed. Acquisition was performed  
192 in resonant mode with the LEICA SP5 CLS by using a laser HeNe at 633nm and a  
193 transmission PMT. The scans were recorded at a 512x512 resolution size and time laps over  
194 200 frames. Their subsequent analysis was realized with the free software ImageJ using the  
195 MtrackJ plugging permitting to determine the average speed in µm/sec of each bacterium  
196 followed. The results reported correspond to mean values obtained from 15 tracking's  
197 measurement for each condition analyzed.

198

### 199 **Time kill study**

200 The effectiveness of Cpd**1** and florfenicol combination against the reference strain CIP 55.110  
201 was determined by the time-kill assay. An overnight culture was diluted to 50-fold into 5 mL  
202 of Columbia agar and grown until the cell suspension reached the mid-logarithmic phase. The  
203 inoculum was then adjusted to 10<sup>7</sup> CFU/mL in Columbia broth supplemented with either, no  
204 addition, sub-inhibitory concentration of florfenicol (MIC/4), sub-inhibitory concentration of  
205 Cpd**1** (7.5 µM) and sub-inhibitory concentration of both. Each tube containing 1 mL was  
206 incubated at 35°C and the bacterial count was determined after several times of incubation: 0,  
207 1, 3, 6, 9, and 12 h by spreading the appropriate dilutions on Columbia agar plates. The plates  
208 were incubated overnight at 35°C before colonies counting. The curves obtained are the result  
209 of two independent experiments.

210

### 211 **Libraries construction for bioinformatic analysis**

212 Libraries were prepared with NebNext kit (New England Biolabs, Ipswich, MA) after Covaris  
213 ME220 Covaris Focused-ultrasonicator (Covaris, Woburn, MA) shearing, according to  
214 manufacturer protocol, and sequenced on a NextSeq550 (Illumina, San Diego, CA) with High  
215 Output 300 cycles V2 cartridge (ref. FC-404-2004). Antibiotic resistance profiles of the four  
216 strains were tested against CARD (22) and oneCodex, with confirmation of phenotypic  
217 resistance except for florfenicol.



## 218 **Results**

219

### 220 **High content screening: selection of florfenicol antibiotic chemosensitizer**

221 The MICs to florfenicol of 10 clinical strains and the reference one were determined, value  
222 ranged from 2 to 32 mg/L showing different level of resistance to this antibiotic (Table 1).

223 A polyamino-isoprenyl chemical library containing 60 molecules has been tested at a  
224 10  $\mu$ M concentration in combination with florfenicol at sub-inhibitory concentration (MIC/4  
225 = 1 mg/L) on the reference strain of *B. bronchiseptica* CIP 55.110 to identify compounds able  
226 to decrease florfenicol MIC below 1mg/L. To avoid compounds with intrinsic activity, a  
227 control experiment was performed in the absence of florfenicol. We then identified  
228 compounds that could only kill bacteria in the presence of florfenicol. Amongst the tested  
229 compounds, only Cpd1 led to an 85% inhibition of the bacterial growth under these  
230 experimental conditions.

231

### 232 **Efficacy of compounds on animal isolates**

233 To extend our study, we evaluated the efficacy of Cpd1 on ten animal isolates. This  
234 compound was able to restore the susceptibility of 8/10 isolates to florfenicol. Nevertheless,  
235 two of them were still resistant to florfenicol alone and in combination (Figure 1). It is  
236 noteworthy that, as already reported in literature (Corbière-Priot and Duménil 1996), a  
237 difference in MICs values was observed, depending on the method used for the evaluation;  
238 the MIC value being higher by using the microdilution broth method with respect to the one  
239 obtained by agar plate method.

240

### 241 **Hit to lead procedure**

242 In the perspective of improving the efficiency of Cpd1, several derivatives were  
243 synthesized (compounds 2-7, Figure 2) as their corresponding water-soluble tartrate salts to  
244 increase their solubility in bacteria growth media and reduce solvent detrimental side-effects.  
245 Compounds 1-4 possess a common farnesyl moiety whereas derivatives 5-7 a geranyl one.  
246 Moreover, each pair Cpd2 / Cpd6, Cp3 /Cpd5 and Cpd4 /Cpd7 have in common the same  
247 polyamine group (Figure 2). The MIC of these compounds were determined for each strain,  
248 no significant intrinsic activity was observed for any of these new compounds (Table 1).

249

### 250 **Checkerboard assay**

251 To determine both the minimal efficient combinations of the designed compounds and  
252 florfenicol that are necessary to inhibit the bacterial growth, we performed a checkerboard  
253 assay on animal isolates, the strain CIP 55.110 being used as control. The results are  
254 summarized in Table 2. Cpd1 exhibited a FIC index of about 0.5 that reflects the strong  
255 synergy between Cpd1 and florfenicol used at MIC/2. We noticed that higher concentrations  
256 are necessary to observe a synergy using florfenicol at MIC/4. We also observed the same  
257 range of FIC index for the farnesyl compounds (Cpd2-Cpd4). On the other hand, we were  
258 unable to determine the FIC indexes for the geranyl ones (Cpd5-Cpd7) due to the high  
259 concentrations needed to inhibit the bacterial growth as mentioned by ND in Table 2.

260

### 261 **Presence of an active efflux in *B. bronchiseptica***

262 For all the strains tested, we observed a MIC for florfenicol ranging from 2 to 4  $\mu$ g/mL  
263 except for two strains 42-F10 and SR11-14 exhibiting a MIC over the resistance breakpoint of  
264 8  $\mu$ g/mL (White *et al.* 2000). We previously reported that efflux mechanisms are produced at  
265 a basal level in Gram negative bacteria (Mamelli *et al.* 2007, Borselli *et al.* 2016) and can  
266 account for the observed low-level of resistance that can be strongly induced when exposed to  
267 toxic compounds (Hay *et al.* 2013, Ghisalberti *et al.* 2005). To evaluate the presence of an

268 active efflux in *B. bronchiseptica*, we measured the ability of the strain CIP55.110 to expel  
269 the 1,2'-dNA, a fluorescent dye which is a substrate of efflux pumps (Bohnert *et al.* 2011).  
270 According to our previous report (Borselli *et al.* 2016), we first observed an accumulation of  
271 the dye after addition of CCCP at a 30  $\mu\text{M}$  final concentration whereas no accumulation was  
272 observed in its absence. This suggested that de-energization of the cytoplasmic membrane by  
273 CCCP blocked dye releasing. Conversely, pre-loaded bacteria incubated with culture media  
274 led to a fluorescence decrease suggesting an active efflux as shown in Figure 3 (black lane)  
275 where the strain CIP55.110 can expel about 75% of the pre-loaded dye. This result allowed us  
276 to compare the ability of the different compounds to inhibit this efflux.

277

### 278 **Action of the derivatives on dye efflux**

279 Some combinations between compounds 1-7 and florfenicol were found to restore  
280 susceptibility of the strains towards this antibiotic. This might suggest that the action of the  
281 compounds contributed to a better accumulation of florfenicol into the bacteria, increasing the  
282 intracellular concentration over the threshold of susceptibility.

283 To understand how the derivatives and especially the farnesyl ones could potentiate  
284 the antibiotic activity, we evaluated their ability to block the efflux activity in *B.*  
285 *bronchiseptica* (Figure 3). An example of the results obtained with compounds used at 100  
286  $\mu\text{M}$  is presented in Figure 3A. As shown on this graph, the strain CIP55.110 is able to expel  
287 the dye (black lane) and this transport is dependent of energy (violet lane) and compounds  
288 exhibit different level of efflux-inhibition. To better compare the efficiency of compounds, we  
289 performed a dose-response assay ranging from 200 $\mu\text{M}$  to 12.5 $\mu\text{M}$ , the results are summarized  
290 in figure 3B. We observed that inhibition mainly concern compounds 1 to 4 and that no  
291 inhibition was found in our conditions, below 25 $\mu\text{M}$ . In addition, we observed a significant  
292 increase of inhibition, from 25 $\mu\text{M}$  to 200 $\mu\text{M}$  for compound 1-4. At 100  $\mu\text{M}$ , the dye efflux  
293 was inhibited by 50% by using Cpd1 and Cpd2 whereas Cpd3 and Cpd4 led to a 30% and  
294 17% efflux inhibition, respectively. Under similar experimental conditions, Cpd5-7 did not  
295 present any significant effect.

296 The other parameter that could modulate the antibiotic intracellular concentration is  
297 the outer-membrane permeability. Thus, we evaluated the permeabilization activity of the  
298 polyamino-isoprenyl derivatives by using previously reported nitrocefin hydrolysis method  
299 (15). We took advantage of the isolate 77-A12 that produces a  $\beta$ -lactamase (not shown).  
300 Interestingly, geranyl compounds (Cpd5-Cpd7) did not exhibit any permeabilization activity  
301 while farnesyl ones (Cpd1-Cpd4) increased the permeability of the outer membrane from 30  
302 to 50% considering that a maximum rate of permeation was obtained by using polymyxin B  
303 (100  $\mu\text{M}$ ) (Figure 4A). However, no influence of the amine structure was detected, Cpd4  
304 (branched amine) presenting a similar activity with respect to the three other farnesyl  
305 compounds bearing a linear amine.

306 In Gram negative bacteria, the PMF drives many of the efflux pumps and inner  
307 membrane transporters, and our compounds are able to inhibit efflux of the fluorescent dye  
308 used (Figure 3). Consequently, it is interesting to determine if the derivatives could alter the  
309 proton motive force across the inner membrane of *B. bronchiseptica*. As shown in figure 4B,  
310 addition of polyamino-isoprenyl derivatives lead to an increase of the level of fluorescence  
311 that reflects the decrease of the proton-motive force. In this case, Cpd1 and Cpd2 cause the  
312 most important depolarization at about 60% of the inner membrane. Surprisingly, derivative  
313 Cpd6 and Cpd4 were also able to depolarize the inner membrane up to 40%. Nevertheless, we  
314 observe a slight effect for Cpd5 and Cp7 which depolarized the inner membrane up to 20%  
315 and 10%, respectively. Molecules responsible for the most important depolarization remained  
316 the farnesyl ones while the geranyl compounds displayed the lower rate of depolarization. In  
317 addition, the presence of a branched amine on the farnesyl moiety greatly influence the

318 activity (compare Cpd1/Cpd4, Cpd2/Cpd4) except for Cpd3, but one must consider that it is  
319 due to the presence of only 3 amino groups instead of Cpd4, as it is encountered for the other  
320 compounds. Thus, conversely to the results obtained from the outer membrane permeation  
321 assays, herein the activity not only depends on the isoprenyl moiety of the compound but also  
322 of the polyamine structure.

323

### 324 **Cpd1 and motility inhibition**

325 As the derivatives can largely impair the proton-motive force and, particularly Cpd1,  
326 we were wondering if this compound could influence the flagella-driven motility that is  
327 highly dependent from the energy triggered by the PMF. We tested here the ability of Cpd1 to  
328 inhibit the swimming and swarming of two strains of *B. bronchiseptica*, the CIP 55.110 which  
329 is susceptible to florfenicol and the SR11-14 which is resistant both to florfenicol and to the  
330 combination florfenicol/Cpd1. Thus, we measured the diameter of the motility halo after 24  
331 hours of incubation at 25°C to assess the functioning of the flagella.

332 The motility of each strain was evaluated in the presence and absence of Cpd1 and/or NaCl by  
333 considering that the flagella could use both the proton and the sodium gradient. We observed  
334 that Cpd1 inhibited the swimming motility at about 40% for both strains (Figure 5A) whereas  
335 a slight effect was encountered towards the swarming motility ranging from 20% and 30%  
336 inhibition depending on the considered strain (Figure 5B). Nevertheless, it is noteworthy that  
337 the swarming inhibition by Cpd1 is stronger in the absence of NaCl (up to 40%, (Figure 5B)).  
338 To further characterize the motility inhibition by Cpd1, we performed a real time assay by  
339 monitoring the motility speed of bacteria after extemporaneous addition of the compound  
340 under the microscope. As shown in figure 5C, a dramatic decrease of speed motility was  
341 observed for both strains. The observed inhibition of motility for both strains CIP55.110 and  
342 SR11-14 correlated with the phenotype observed previously, strongly suggests that Cpd1  
343 inhibits flagella motilities by disrupting the PMF.

344

### 345 **Time effect on bacterial growth**

346 To further characterize the Cpd1/florfenicol combination, its *in vitro*  
347 pharmacodynamic (PD) parameters were evaluated using time-kill kinetics assays (Fig 6).  
348 Curves were obtained by plotting the number of CFU at every time point for the synergistic  
349 concentrations of the drugs.

350 As expected, the use of florfenicol at a sub-inhibitory concentration (MIC/4) had no  
351 effect on the cell growth such as the Cpd1 used at a concentration of 7.5 µM which  
352 corresponds to its MIC/32. Nevertheless, the combination of both totally inhibits the cell  
353 growth and, after 9 hours of incubation, the reduction of bacterial cell count was around two  
354 Log compared to the bacteria incubated with florfenicol or Cpd1 alone. After 12 hours of  
355 incubation, the cell count was still reduced of four Log demonstrating a significant effect of  
356 the combination compared to the same antimicrobials administered alone (Figure 6). The  
357 combination florfenicol/Cpd1 appeared to be bacteriostatic as florfenicol used alone.

358

### 359 **Florfenicol resistance mechanism investigation**

360 Two of the strains included in our study (42F10 and SR11-14) exhibited resistance to  
361 florfenicol (MIC = 8 and 32 µg/mL, respectively). Such resistance is not common but has  
362 already been described and the *floR* gene was identified in some isolates as responsible for  
363 this resistance (Kadlec *et al.* 2007, Kadlec *et al.* 2018). To check if *floR* could be associated  
364 with florfenicol resistance in these two strains, we designed primers to amplify the gene  
365 according to the sequence previously described in the study of Kadlec (Kadlec *et al.* 2007).  
366 No amplification was detected despite several attempts suggesting that another mechanism of  
367 resistance was involved in these strains. Taken together, our data suggested that another

368 mechanism was involved in florfenicol resistance in these two strains. We thus decided to  
369 determine the complete sequenced genomes of those two strains, as well as two other strains,  
370 our reference CIP55-110 and 77A12 which was resistant to  $\beta$ -lactams, were realized (SRA  
371 accession: PRJNA551886).

372 In order to be able to highlight a new gene of resistance or a *floR* derived one, we mapped our  
373 two libraries made from resistant strains against reference strain *B. bronchiseptica* 253, by  
374 using BWA MeM software (version 0.7.16a-r1181) with a minimum seed length of 28, all  
375 other options kept to standard. Owing the fact that this strain does not have resistance to  
376 florfenicol, we selected reads that did not map to this strain. We reconstructed *de novo* the  
377 two other strains CIP55-110 and 77H12 with SPAdes version 3.9.1 (Bankevich *et al.* 2012)  
378 and we mapped the remaining reads to the obtained contigs with the same goal. We ended  
379 with 581 k-reads and 377 k-reads for SR11-14 and 42F10, respectively (Supplemental  
380 Material). We reconstructed *de novo* contigs with those remaining reads, keeping in mind the  
381 hypothesis that genes responsible for florfenicol resistance would be present in such contigs.  
382 We ended with 416 contigs and 331 contigs, respectively, that were mapped against all the  
383 florfenicol and/or chloramphenicol resistance associated genes published until now (634  
384 sequences). Unfortunately, we found any match.

385 As a last attempt, considering that protein sequence is better preserved than nucleotide  
386 sequence, we performed a blastx of all our contigs against all the available florfenicol  
387 resistance related protein sequences but even there unsuccessfully. Genes associated with  
388 resistance to antibiotics specifically detected in those two strains were found, indicating that  
389 our pipeline was able to detect genes present only in those strains and not in the reference and  
390 non-resistant ones (*sul2* in SR11-14 and TEM-116 in 42F10). Interestingly, genes sharing  
391 homology with ABC transporters and OptrA (Tamang *et al.* 2017), that have been described  
392 to be associated with florfenicol resistance in *Enterococcus faecium* have been detected co-  
393 localized on same contigs, that also exhibit plasmid associated features. Interestingly, OptrA  
394 was shown to afford cross-resistance to chloramphenicol and florfenicol in *E. faecium* (Wang  
395 *et al.* 2015, Bender *et al.* 2018, Na *et al.* 2019) that can be correlated with the cross-resistance  
396 to these two antibiotics in the two strains studied herein (see Supplementary Table 1).

397 **Discussion**

398 The international and European authorities requested to use with parsimony antibiotics  
399 which can favor emergence of resistance in human and veterinary medicine. For the first time,  
400 an alternate way is studied by using adjuvant molecules that enhance florfenicol antibiotic  
401 activity. This strategy allowed us to decrease by four the MIC of the majority of strains tested  
402 here with small amounts of polyamino-isoprenyl derivatives, previously known to potentiate  
403 antibiotics by acting on membrane functions (Blanchet *et al.* 2016). Thus, even if florfenicol  
404 is only used to treat animal infections it presents cross resistance with chloramphenicol and it  
405 appears important to prevent the spread to human pathogens.

406 Today, it is recognized that human health is directly related to animal health and the  
407 environment. The dramatic increase in antibiotic resistance and the scarcity of new drugs on  
408 the market in recent decades highlight the threat of moving to the past era of antibiotics, with  
409 a significant increase in infection mortality. In this context, taking into account the "One  
410 Health" problem becomes an urgent need. This must allow collaborative efforts between  
411 health actors, but also breeders and those involved in environmental management.

412 This includes the involvement of a research at the interface between chemistry and  
413 microbiology, to develop new infection control strategies, by developing molecules that target  
414 specific new bacterial processes and / or new molecules that block / inhibit resistance  
415 mechanisms already under way. In recent decades, the efflux mechanisms have become  
416 increasingly important for human and animal health, we propose to block the mechanisms of  
417 resistance by efflux of Gram-negative bacteria.

418 According to our previous work (Borselli *et al.* 2016), we evaluated enhancing activity  
419 of our polyamino-isoprenyl chemical library (used at a 10  $\mu$ M concentration) in the presence  
420 of florfenicol at a sub-inhibitory concentration against *B. bronchiseptica*, CIP 55.110  
421 reference strain. Thus, Cpd1 was identified as an interesting chemosensitizer of *B.*  
422 *bronchiseptica* to florfenicol and evaluated against ten animal isolates leading to the  
423 restoration of susceptibility for 8 of these 10 strains. Two isolates were resistant to the  
424 combination, and the mechanism of resistance involved remains unknown.

425 Subsequently, a pharmacomodulation study was performed to try to improve the  
426 encountered activity. In this context, six derivatives, three geranyl ones and their  
427 corresponding farnesyl homologues were prepared and evaluated in a checkerboard assay.  
428 First, we noticed that Cpd1 remained the better tested compound needing the lowest  
429 concentration to potentiate florfenicol activity. It is also noteworthy than all farnesyl  
430 compounds showed better efficiency than geranyl ones. As polyamines are well known agents  
431 to disrupt membranes (Blanchet *et al.* 2016), we investigated the effect of the compounds on  
432 membranes physiology and stability. We investigated the permeability of the outer membrane  
433 which play a key role in antibiotic accumulation into Gram-negative bacteria and as expected,  
434 all the tested polyamino-isoprenyl compounds increase the outer membrane permeability,  
435 farnesyl compounds being better permeabilizers than geranyl ones.

436 The presence of an active efflux in *B. bronchiseptica* was demonstrated by using  
437 CCCP allowing an efficient dye loading into the bacterial membranes. These results  
438 corroborate the genome sequence analysis from *B. pertussis* (a human and animal pathogen  
439 closely related to *B. bronchiseptica*) suggesting the presence of putative RND efflux pumps  
440 coding sequences (Andrade *et al.* 2014). To our knowledge, it is also the first time that efflux  
441 was determined in *B. bronchiseptica*. Thereby, we evaluated the effect of Cpd1 and its  
442 derivatives on the efflux activity of *B. bronchiseptica*. The molecules able to inhibit the dye  
443 efflux were the farnesyl ones while the geranyl derivatives demonstrated no ability to block  
444 the efflux activity (Cpd1 and Cpd4 leading to 50 % inhibition (used at 100  $\mu$ M)). Moreover,  
445 we observed a dose response effect for each farnesyl compound as encountered in a previous  
446 work on *Pseudomonas* (Borselli *et al.* 2016). It also appeared that the farnesyl moiety was

447 essential to obtain high activities. All these data led us to envision that these compounds could  
448 act against PMF, which is required for many cell processes, as efflux pump systems but also  
449 the flagella motors.

450 The flagella that potentially contribute to virulence, could be considered as a new  
451 target for the development of motility inhibitors to circumvent the spread of bacterial  
452 infections (Malapaka *et al.* 2007). Effect of Cpd1 was studied and a significant inhibition on  
453 the flagella-dependent motilities was observed. Nevertheless, since *B. bronchiseptica* can also  
454 use the sodium gradient, we were unable to discriminate if Cpd1 targets preferentially the  
455 proton or sodium gradient. We concluded that this effect is probably due to a general  
456 inhibition. On the other hand, we noticed that the inhibition of motility occurred in an  
457 independent way of the resistance level of the strain. Indeed, a resistant strain (SR11-14) and  
458 the reference strain CIP55.110 gave the same response to inhibition of motility after treatment  
459 with Cpd1. More interestingly, the study of the resistance mechanism of these strains by  
460 bioinformatic investigations showed the presence of a gene sharing homologies with the gene  
461 coding the ABC-F type ribosomal protection protein OptrA, known to confer florfenicol and  
462 chloramphenicol resistance in *Enterococcus faecium* and to use ATP as a source of energy  
463 (Wang *et al.* 2015, Bender *et al.* 2018, Na *et al.* 2019). This is consistent with our results  
464 demonstrating that Cpd1 targets the PMF and correlates with the absence of efficacy towards  
465 the strains exhibiting this mechanism of resistance. In the strain SR 11-14 one might consider  
466 that OptrA and/or efflux pumps are overexpressed in this strain. We can hypothesize that  
467 florfenicol resistance could have been acquired through horizontal transfer from  
468 *Enterococcus*, and this hypothesis is strengthened by the fact that the contig containing  
469 resistance associated genes showed high homology with plasmids found in such bacteria.

470 Finally, to better characterize the antibiotic/derivative combination we did time kill  
471 assays at the concentrations which have shown synergistic effect. This florfenicol/Cpd1  
472 combination reduces the antibiotic efficient concentration four times and so could allow to  
473 reduce drastically the amount of the antibiotic used. Because Cpd1 did not modify the  
474 bacteriostatic activity of florfenicol, but increased its efficiency, one may consider that Cpd1  
475 allows an increase of the intra-cellular concentration of the antibiotic (Vergalli *et al.* 2018).  
476 Experiments are in progress to evaluate this hypothesis and results will be reported in due  
477 course.

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### **Conflict of interest Statement**

The author(s) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### **Authors contribution statement**

All authors conceived the approach; DB carried out all the experiments related to bacteria; JM Brunel carried out the synthesis, purification and analyses of the compounds; OG performed the DNA sequencing and the sequence analyses; DB, JM Brunel and JM Bolla wrote the main manuscript text; DB and JM Brunel prepared the figures; all authors reviewed the manuscript.

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607 **Figures legend**

608

609 Figure 1: The ability of *B. bronchiseptica* reference strain CIP55,110 (5  $\mu$ L of  $5 \times 10^5$  CFU  
610 were spotted on the plates) to grow under various experimental conditions was assessed in  
611 solid media containing (left to right) no compound, Cpd1 (compound 1) (10  $\mu$ M), FF  
612 (Florfenicol) (2 $\mu$ g/mL), FF (2 $\mu$ g/mL) + Cpd1 (10  $\mu$ M).

613

614 Figure 2: Structure of farnesyl and geranyl polyamine derivatives Cpd1-Cpd7.

615

616 Figure 3: Inhibition of the efflux of the 1,2' dinaphthylamine dye by derivatives 1-7. 3A.  
617 Efflux was triggered after 200s by the addition of culture media. The intensity of fluorescence  
618 emission for 1,2' dinaphthylamine is given in relative fluorescence units (RFU). Each  
619 compound was tested at 100  $\mu$ M concentration; as controls an experiment without addition of  
620 compounds and culture media (purple curve) was also included. 3B. Percentage of efflux  
621 inhibition obtained for each compound. Error bars represent standard deviations of 3  
622 independent biological experiments.

623

624 Figure 4: Effect of polyamine derivatives on membrane functions 4A) Outer membrane  
625 permeabilization by the different compounds at 100  $\mu$ M. Nitrocefin hydrolysis was followed  
626 at 490nm. 4B) Inner membrane depolarization measured by the increase fluorescence of  
627 DisC<sub>3</sub>(5) in the presence of the different compounds at 100  $\mu$ M. Error bars represent standard  
628 deviations of 3 independent biological experiments.

629

630 Figure 5: Effect of 10  $\mu$ M of the hit compound on bacterial motility of a susceptible strain  
631 (CIP55.110) and a florfenicol resistant isolate (SR11-14) +/-NaCl. Graph represent the  
632 percentage of inhibition of the motility halo in presence of Cpd1. 5A) Picture taken after 24h  
633 incubation of the swimming plate. 5B) Swarming motility, halo measured after 48 h  
634 incubation. 5C) Picture taken after 24h of incubation of swimming plate with NaCl inoculated  
635 with SR11-14, containing no compound (left) and 10  $\mu$ M of Cpd1 (right). In all cases, error  
636 bars represent standard deviations of 3 independent experiments.

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638 Figure 6: Time-kill curves of florfenicol (FF) (0.5 mg/L), Cpd1 (7.5  $\mu$ M) and of the  
639 combination FF (0.5 mg/L)+Cpd1 (7.5  $\mu$ M) over a 12 hours period against strain CIP55.110.  
640 Errors bars represent standard deviations of 2 independent biological experiments.

641 **Table 1:** Susceptibilities to florfenicol (FF) and polyamine derivatives (**Cpd1-Cpd7**) of  
 642 various *B. bronchiseptica* strains.  
 643

Strains	Source	MIC	MIC ( $\mu\text{M}$ )						
		( $\mu\text{g/mL}$ )	FF	Cpd1	Cpd2	Cpd3	Cpd4	Cpd5	Cpd6
<b>CIP55.110</b>	Pasteur Institute	4	187.5	50	50	50	>750	>750	375
<b>42-F10</b>	Labofarm	8	187.5	50	50	50	>750	>750	375
<b>48-J7</b>	Labofarm	4	187.5	50	100	50	>750	>750	375
<b>77-C4</b>	Labofarm	4	187.5	50	100	100	>750	>750	375
<b>62-H4</b>	Labofarm	4	187.5	50	50	50	>750	>750	375
<b>73-B14</b>	Labofarm	2	187.5	50	100	100	>750	>750	375
<b>67-A11</b>	Labofarm	4	187.5	50	100	50	>750	>750	375
<b>78-E4</b>	Labofarm	4	187.5	50	100	50	>750	>750	375
<b>77-A12</b>	Labofarm	4	187.5	50	100	50	>750	>750	375
<b>SR11-24</b>	Labofarm	2	187.5	50	50	50	>750	>750	375
<b>SR11-14</b>	Labofarm	32	187.5	50	50	50	>750	>750	375

644

645 **Table 2:** FIC index obtained for each compound tested leading to a decrease of florfenicol  
 646 concentration of 50% and 75% respectively, under conditions A and B. Condition A (MIC/2).  
 647 Condition B (MIC/4). ND (Not determined).  
 648  
 649

	Cpd1		Cpd2		Cpd3		Cpd4		Cpd5		Cpd6		Cpd7	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
<b>CIP 55.110</b>	<b>0.50</b>	0.29	0.56	0.75	0.56	0.50	0.63	0.38	0.53	ND	0.50	ND	0.53	ND
<b>42-F10</b>	<b>0.51</b>	ND	0.56	1.25	0.53	0.75	0.63	0.75	0.63	ND	0.57	ND	0.63	ND
<b>48-J7</b>	<b>0.54</b>	0.33	0.75	1.25	0.63	0.75	1.00	1.25	0.77	ND	0.63	ND	0.63	ND
<b>77-C4</b>	<b>0.52</b>	ND	0.56	0.75	0.53	1.25	0.63	1.25	0.57	ND	0.52	ND	0.57	ND
<b>62-H4</b>	<b>0.54</b>	ND	1.00	0.50	0.75	1.25	0.75	1.25	0.77	ND	ND	ND	ND	ND
<b>73-B14</b>	<b>0.51</b>	ND	0.63	0.75	0.53	0.75	0.63	1.25	ND	ND	0.63	ND	ND	ND
<b>67-A11</b>	<b>0.50</b>	0.26	0.53	0.50	0.52	0.38	0.53	0.50	0.63	ND	0.50	0.28	0.57	ND
<b>78-E4</b>	<b>0.51</b>	ND	0.75	0.25	0.52	0.75	0.53	0.50	0.51	0.38	0.50	0.38	0.57	ND
<b>77-A12</b>	<b>0.50</b>	0.26	0.53	0.50	0.52	0.50	0.53	0.50	0.52	0.32	0.52	0.32	0.57	ND
<b>SR11-24</b>	<b>0.52</b>	ND	0.75	0.75	0.53	1.25	0.56	0.75	0.63	ND	0.57	ND	0.57	ND
<b>SR11-14</b>	<b>0.51</b>	ND	0.63	0.75	0.56	0.75	1.00	0.75	0.63	ND	0.57	ND	0.57	ND

650