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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 3rd and
46 4th 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 (DiSC₃(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 5x10⁵ 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 5x10⁵ 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 CH₂Cl₂/MeOH/ NH₄OH (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 μ L) of 5×10^5 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 alone . The antibacterial combination was considered synergistic when the FICI was ≤ 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 β -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 MgCl₂ (PPB) supplemented with 30 μ 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 μ L of compound derivatives
139 dilutions already set up in microplates, to give final concentrations ranging from 1.56 to 200
140 μ M. Then, 50 μ L of nitrocefin was added to obtain a final concentration of 50 μ 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 μ 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 μ M final
151 concentration, supplemented with the proton dissipator CCCP 30 μ 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 μ L of the
154 desired tested compounds were mixed with 100 μ L of the cell suspension yielding final
155 concentration ranging from 12.5 to 200 μ M. The dye transport was triggered after addition of
156 5 μ 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 μ L of a 160 μ M solution of DiSC₃(3) to 100
170 μ L of the cell suspension to reach a final concentration of 7.6 μ M. After a period of
171 incubation (16.5 min) to allow the dye incorporation into the polarized membranes, 10 μ L 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 μ M 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 μ m/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 10⁷ 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 μ M) 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 μ 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 μ g/mL
263 except for two strains 42-F10 and SR11-14 exhibiting a MIC over the resistance breakpoint of
264 8 μ 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 μM 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 μM 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 μM to 12.5 μM , 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 μM . In addition, we observed a significant
292 increase of inhibition, from 25 μM to 200 μM for compound 1-4. At 100 μM , 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 β -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 μM) (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 β -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 μ 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 μ 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 μ L of 5×10^5 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 μ M), FF
612 (Florfenicol) (2 μ g/mL), FF (2 μ g/mL) + Cpd1 (10 μ 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 μ 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 μ M. Nitrocefin hydrolysis was followed
626 at 490nm. 4B) Inner membrane depolarization measured by the increase fluorescence of
627 DisC₃(5) in the presence of the different compounds at 100 μ M. Error bars represent standard
628 deviations of 3 independent biological experiments.

629

630 Figure 5: Effect of 10 μ 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 μ 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 μ M) and of the
639 combination FF (0.5 mg/L)+Cpd1 (7.5 μ 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 (μM)						
		($\mu\text{g/mL}$)	FF	Cpd1	Cpd2	Cpd3	Cpd4	Cpd5	Cpd6
CIP55.110	Pasteur Institute	4	187.5	50	50	50	>750	>750	375
42-F10	Labofarm	8	187.5	50	50	50	>750	>750	375
48-J7	Labofarm	4	187.5	50	100	50	>750	>750	375
77-C4	Labofarm	4	187.5	50	100	100	>750	>750	375
62-H4	Labofarm	4	187.5	50	50	50	>750	>750	375
73-B14	Labofarm	2	187.5	50	100	100	>750	>750	375
67-A11	Labofarm	4	187.5	50	100	50	>750	>750	375
78-E4	Labofarm	4	187.5	50	100	50	>750	>750	375
77-A12	Labofarm	4	187.5	50	100	50	>750	>750	375
SR11-24	Labofarm	2	187.5	50	50	50	>750	>750	375
SR11-14	Labofarm	32	187.5	50	50	50	>750	>750	375

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

650