1 Drug screening to identify compounds to act as co-therapies 2 for the treatment of pathogenic *Burkholderia*

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4 Sam Barker,^a Sarah V. Harding,^b David Gray,^c Mark I. Richards,^b Helen S.
5 Atkins,^{a,b} Nicholas Harmer^{a,d†}

6 ^aDepartment of Biosciences, College of Life and Environmental Sciences,
7 University of Exeter, Exeter, United Kingdom; ^bDefence Science and
8 Technology Laboratory, Porton Down, Salisbury, United Kingdom; ^cDrug
9 Discovery Unit, University of Dundee, Scotland; ^dLiving Systems Institute,
10 Stocker Road, Exeter, United Kingdom.

11
12 [#]Current address: Phoremest Ltd, Building 250, Babraham Research Campus

13 [†]To whom correspondence should be addressed:

14 Living Systems Institute, Stocker Road, Exeter EX4 4QD, U.K.

15 N.J.Harmer@exeter.ac.uk

17 Abstract

18 *Burkholderia pseudomallei* is a soil-dwelling organism present throughout the
19 tropics, and is the causative agent of melioidosis, a disease that is believed to
20 kill 89,000 people per year. It is naturally resistant to most currently available
21 antibiotics. The most efficacious treatment for melioidosis requires at least two
22 weeks of intravenous treatment with ceftazidime or meropenem. This places a
23 large treatment burden on the predominantly middle income nations where the

majority of disease occurs. We have established a high-throughput assay for compounds that could be used as a co-therapy to potentiate the effect of ceftazidime, using the related non-pathogenic bacterium *Burkholderia thailandensis* as a surrogate. Optimization of the assay gave a Z' factor of 0.68. We screened a library of 61,250 compounds, and identified 29 compounds with a pIC_{50} ($-\log_{10}(IC_{50})$) greater than five. Detailed investigation allowed us to down select to six “best in class” compounds, which included the licensed drug chloroxine. Co-treatment of *B. thailandensis* with ceftazidime and chloroxine reduced culturable cell numbers by two orders of magnitude over 48 hours compared to treatment with ceftazidime alone. Hit expansion around chloroxine was performed using commercially available compounds. Minor modifications to the structure abolished activity, suggesting that chloroxine likely acts against a specific target. Finally, preliminary data also demonstrates the utility of chloroxine to act as a co-therapy to potentiate the effect of ceftazidime against *B. pseudomallei*. This approach successfully identified potential co-therapies for a recalcitrant Gram-negative bacterial species. Our assay could be used more widely to aid in chemotherapy against these bacteria.

41

Keywords: Antimicrobial, PrestoBlue, *Burkholderia thailandensis*, *Burkholderia pseudomallei*, High throughput screening, Gram-negative.

44

45

46 Introduction

47 ***Burkholderia pseudomallei*** is the causative agent of melioidosis, a disease
 48 endemic to many regions across the tropics [1]. It is believed to cause
 49 approximately 89,000 deaths per annum worldwide [2, 3], with the large majority
 50 of the burden falling on less developed or lower middle income countries.
 51 Melioidosis can present in many ways, which significantly complicates diagnosis
 52 [4]. Clinical presentations include skin infections, suppurative parotitis,
 53 genitourinary infections, and pneumonia [5]. The most serious infections can
 54 develop to sepsis, and abscesses on internal organs are common [1, 6]. In the
 55 absence of treatment, mortality from acute infections is high; even with
 56 treatment, mortality approaches 40% in many affected areas [7]. Patients with
 57 access to adequate diagnosis and treatment facilities have reduced mortality
 58 rates due to developments in treatment [8], currently consisting of an intensive
 59 treatment phase of intravenous ceftazidime or meropenem for at least 14 days,
 60 followed by oral eradication therapy with trimethoprim-sulfamethoxazole lasting
 61 between 3 and 6 months [1, 9, 10] . The cost of this treatment regime is high
 62 and the burden of disease in least developed countries (e.g. Cambodia) may
 63 prevent those in need from being treated [11, 12].

64

65 *B. pseudomallei* is found in soil and water, preferring anthrosol and acrisol soil
 66 types [2, 3]. Like many *Burkholderia*, it is an opportunistic pathogen of humans,
 67 and most patients have at least one pre-disposing risk factor (with diabetes
 68 mellitus the most common) [13]. In the host, *B. pseudomallei* generally adopts
 69 an intracellular lifestyle, and can invade and replicate in a range of cell types

70 [14], with neutrophils being particularly preferred in mice [15]. *B. pseudomallei*
 71 has multiple virulence factors that support this lifestyle; the intracellular niche
 72 provides the organism with protection against the host immune system. This is
 73 particularly important as the humoral response is the best correlate of protection
 74 in vaccination studies [16]. The intracellular location also makes antibiotic
 75 chemotherapy more challenging as compounds must cross an additional
 76 biological membrane.

77

78 *B. pseudomallei* is naturally resistant to most clinically used antibiotics,
 79 including some of the more recently developed antibiotics [1, 17]. The most
 80 effective treatment is with an intensive phase of at least two weeks intravenous
 81 ceftazidime and/or meropenem [18]; increasing this phase to at least four weeks
 82 has reduced the rate of relapse. However, in many lower income settings
 83 alternative eradication regimes are used that have increased relapse rates [19].
 84 When cultured to stationary phase or in hypoxic conditions, most *Burkholderia*
 85 species show a high subpopulation that are recalcitrant to antibiotic treatment
 86 [20]. This observation is believed to mimic behavior *in vivo*, with *B.*
 87 *pseudomallei* surviving in biofilms or intracellular niches where cellular
 88 conditions promote antibiotic tolerance [21-23]. This can then lead to recurrent
 89 or latent forms of the disease and the relapse of infections in humans where
 90 longer term antibiotic treatment is not administered [24]. Although significant
 91 progress has been made towards a melioidosis vaccine, candidates have yet to
 92 enter clinical trials [25, 26].

93 This presents an urgent unmet need for affordable novel drugs that supplement
 94 current effective therapeutics to reduce the cost and duration of treatment and
 95 to prevent relapse of infection [18, 27]. We hypothesized that small molecules
 96 could act as co-therapies that could be administered alongside front-line
 97 treatments with the aim of reducing the rates of recurrent infection. We aimed to
 98 develop an assay that would allow rapid screening of a compound library to
 99 identify and validate such compounds, as a step towards a potential therapy. As
 100 *B. pseudomallei* is a Containment Level 3 bacterium, *Burkholderia thailandensis*
 101 was selected for this study. This is a close relative of the pathogenic *B.*
 102 *pseudomallei* with over 85% gene conservation [28]. As *B. thailandensis* does
 103 not cause disease in immunocompetent humans [28-30], it has been classified
 104 as a Containment Level 2 bacterium, and is commonly used as a surrogate for
 105 *B. pseudomallei*. Previous studies have shown that approximately 0.1% of *B.*
 106 *thailandensis* cells survive for 24 hours following treatment with 100X MIC of the
 107 front-line antibiotic ceftazidime *in vitro* [20].

108
 109 A phenotypic assay using the cell viability reagent PrestoBlue™ was used to
 110 screen compounds from a diversity library containing nearly 5,000 core
 111 fragments [31] at the Drug Discovery Unit (DDU) in Dundee
 112 (<http://www.drugdiscovery.dundee.ac.uk/>). Primary screening identified
 113 compounds that were active as co-therapies to potentiate the effect of
 114 ceftazidime hydrate against *B. thailandensis*. Following hit confirmation and
 115 potency determination, six compounds with IC₅₀ values < 10 µM were taken
 116 forward in the study. We identified two compounds, **A**: 5,7-dichloroquinolin-8-ol

117 (also known as chloroxine) and **B**: 5-ethyl-5'-(2-hydroxyphenyl)-3'H-spiro[indole-
118 3,2'-[1,3,4]thiadiazol]-2(1H)-one (Figure 1), as promising hits for this indication.

119 Results

120

121 Assay development

122 The aim of this study was to identify compounds that may have use as co-
 123 therapies for the treatment phase of melioidosis. We aimed to develop an assay
 124 that would identify compounds that reduced the proportion of *B. thailandensis*
 125 cells that remained viable when delivered in combination with ceftazidime
 126 hydrate (400 µg/ml, 100X MIC; large excess of MIC is generally used for assays
 127 of antibiotic tolerant cell populations, e.g. [32-35]). We investigated a range of
 128 absorbance, fluorescence, luminescence and qPCR based assays for
 129 correlates of cell viability and evaluated the assay effectiveness using the Z'
 130 statistic [36] commonly used for high-throughput screening (HTS) [37]. The
 131 requirement of this initial assay was to distinguish a ceftazidime treated culture
 132 from the negative controls with a Z' score greater than 0.4. A phenotypic cell
 133 viability assay using the resazurin-based reagent PrestoBlue met this criterion,
 134 resulting in a greater Z' score than the alternative assays (details of other
 135 approaches investigated are described in Supplementary results). This offered
 136 the opportunity for high throughput screening in a convenient and affordable
 137 manner with good discrimination for the reduction in the survival of *B.*
 138 *thailandensis*. A *B. thailandensis* culture, diluted in two-fold steps, and treated
 139 with ceftazidime hydrate to simulate increasing levels of cell death,
 140 demonstrated a clear reduction in the resazurin reduction signal when fewer
 141 cells were present initially, indicating that the color change observed reflected
 142 surviving cell numbers (Figure 2). The assay was then optimized for use in 384

143 well plates with automated dispensing of reagents. The final assay quality was
 144 determined, comparing *B. thailandensis* at the optimized test cell density in M9
 145 media supplemented with ceftazidime hydrate, to heat killed bacteria in the
 146 same media. The final assay resulted in a Z' score of 0.68 (Supplementary
 147 Figure 1), which is consistent with an HTS requirement for a score of 0.5-0.7.
 148 Controls included in the HTS protocol indicated that this assay quality was
 149 maintained throughout the high throughput screening.

150

151 **High Throughput Screening**

152 Primary single point screening took place for the 61,250 compounds comprising
 153 the DDU's Diversity screening library [31]. As expected, the majority of the
 154 compounds were inactive (Figure 3), with some compounds showing compound
 155 effects on the assay (indicated by the tail of compounds showing >150% of the
 156 mean fluorescence). Using the median percentage effect plus three standard
 157 deviations, a statistically significant cut-off of 34.3% inhibition was determined.
 158 This identified 2,127 unique compounds as 'hits'. This exceeded the capability
 159 for downstream analysis. As a result, a pragmatic cut off of 45% inhibition was
 160 selected (Figure 3, red arrow), resulting in 345 unique compounds. Some of
 161 these were down-selected due to known promiscuity issues. We selected 309
 162 compounds for detailed screening: these included some near analogues to hits
 163 from within the DDU collection that showed high activity and good
 164 physiochemical properties. For these 309 compounds, a ten point, 2-fold,
 165 concentration response assay was performed in duplicate (Supplementary
 166 figure 2) with criteria for a positive hit set as greater than 50% inhibition at the

167 highest concentration tested (100 μ M). Acceptable concentration response
168 relationships were returned for 58 compounds, of which 29 showed a pIC_{50} (-
169 $\log_{10}(IC_{50})$) values > 5 , indicating at least 50% activity at 10 μ M and that the
170 compound is a potential “hit”.

171

172 **Down selecting compounds**

173 Concentration dependent killing assays were repeated for the 29 compounds
174 selected using newly sourced stocks of the same compounds and performed in
175 triplicate over a larger range of concentrations. pIC_{50} , hillslope and maximal
176 effect were used to further down select to six compounds (**A-F**), all of which
177 displayed a confirmed $pIC_{50} > 5$ (Figure 4). At this point a secondary assay
178 using the nucleic acid stain SYTO9 to quantify surviving cells was used to
179 confirm hits, particularly for those compounds where signal interference was
180 observed in the PrestoBlue assay (Figure 5). Two compounds, chloroxine (5,7-
181 dichloroquinolin-8-ol, **A**) and 5-ethyl-5'-(2-hydroxyphenyl)-3'H-spiro[indole-3,2'-
182 [1,3,4]thiadiazol]-2(1H)-one (**B**; Figure 1) were selected as excellent
183 representatives of compounds effective in the two assays.

184

185 **Minimum Inhibitory Concentration (MIC)**

186 The experiments described above highlighted compounds that had activity on
187 cells that had survived following ceftazidime hydrate treatment. Our hypothesis
188 was that these compounds either potentiated the effects of ceftazidime hydrate,
189 or were toxic to cells in a metabolic state that rendered them insensitive to
190 ceftazidime hydrate. However, we reasoned that they might be acting as

191 antibiotics in their own right. We therefore determined the MIC for all six
192 compounds. Chloroxine demonstrated antimicrobial activity, with an MIC of 4
193 $\mu\text{g/mL}$ (compared with 4-8 $\mu\text{g/mL}$ for ceftazidime). This was not unexpected, as
194 chloroxine is known to be an effective antimicrobial with activity described
195 against a range of Gram-positive bacteria and fungi. Compounds **B-F** showed
196 no inhibitory effect on bacterial growth when used as sole treatment at
197 concentrations up to 1 mM (data not shown). Chloroxine and ceftazidime
198 showed no evidence of synergistic antimicrobial effects (Supplementary Figure
199 3), suggesting that the effects observed reflect potentiation of the ceftazidime
200 effect on tolerant cells.

201

202 **Time dependent killing**

203 A time dependent killing assay was performed to demonstrate that the effect of
204 chloroxine is complementary to ceftazidime. Stationary phase cells were
205 resuspended in fresh media supplemented with ceftazidime hydrate (100X
206 MIC), with or without 50 μM of chloroxine. Colony forming units were
207 determined over 48 hours of incubation. Chloroxine significantly reduced the
208 number of viable cells after 24 hours when compared to treatment with
209 ceftazidime hydrate alone or chloroxine alone ($p < 0.05$, and $p < 0.005$
210 respectively), with a reduction in cell number by nearly two orders of magnitude
211 at 48 hours (Figure 6). This validates the activity of chloroxine. We also
212 performed a cytotoxicity assay that demonstrated that chloroxine was not toxic
213 to mammalian cells (Supplementary Figure 4).

214

215 Hit Expansion

216 One possibility was that chloroxine was acting non-specifically as an oxidizing
217 agent. Hit expansion using similar commercially available compounds was
218 therefore carried out to identify the neighboring structures of the hit compounds
219 to gain insight into the structure-activity relationship. This may also assist in the
220 future development of this compound from hit to lead.

221

222 Chloroxine (**A**: Figure 1) is a relatively small synthetic compound with limited
223 scope for improvement. The pIC_{50} was determined as 5.5 using the PrestoBlue
224 assay (Figure 4). A total of eleven similar compounds were commercially
225 available, and were used for this screen. None of these demonstrated
226 increased potency in the assay, as we had expected given the limited selection.
227 However, the pattern of loss of potency provides clear insights into how
228 chloroxine could be further modified. It was clear that the identity of the
229 substituent at the 7-position was important. Replacement of this with an amino
230 group significantly reduced activity (pIC_{50} reducing to 3.4; Figure 7A). Similarly,
231 addition of a methyl group at the 2-position was poorly tolerated, leading to a
232 loss of detectable activity at the concentrations tested (Figure 7B). The
233 halogens in the compound could also be altered to some extent. Replacement
234 of the chlorine atom with bromine at the 7-position was tolerated, but only if the
235 chlorine in the 5-position was also removed (reduction in pIC_{50} from 5.5 to 5.1,
236 Figure 7C). Two alternative structures with bromine were not active
237 (Supplementary Table 1). Iodide ions were also tolerated in place of the
238 chlorines, again with a small loss of activity (Supplementary Table 1). More

239 extensive alterations to the structure of chloroxine resulted in the loss of at least
240 one order of magnitude of activity (Supplementary Table 1).

241

242 Finally, to validate the use of *B. thailandensis* as a proxy for *B. pseudomallei*,
243 we repeated the original PrestoBlue assay with ceftazidime hydrate and
244 chloroxine against *B. pseudomallei*. The fluorescent signal seen for *B.*
245 *pseudomallei* was approximately double the *B. thailandensis* signal; this is
246 unlikely to be significant as fluorescence observed is known to vary from
247 species to species with this reagent [38]. Chloroxine demonstrated a similar
248 level of activity against *B. pseudomallei* to that seen against *B. thailandensis*
249 (Figure 8). The IC₅₀ observed against *B. pseudomallei* was slightly higher than
250 for *B. thailandensis*. This result suggests that our assay could be used with *B.*
251 *pseudomallei*.

252

253 Discussion

254 This study aimed to identify compounds that were effective in reducing the
 255 proportion of *Burkholderia* cells that survive following treatment with supra-lethal
 256 concentrations of ceftazidime. Ceftazidime is a front-line therapy for melioidosis
 257 [1], the current best practice stating administration for two weeks of intravenous
 258 treatment to minimize the risk of relapse [18]. Ceftazidime specifically targets
 259 the penicillin-binding protein 3 BPSS1219 in *B. pseudomallei* [23, 39]; the *B.*
 260 *thailandensis* orthologue shows 97% identity at the amino acid level.
 261 Ceftazidime treatment at sub-MIC concentrations induces filamentation of *B.*
 262 *pseudomallei*, whilst ceftazidime is lytic at higher concentrations [40]. Our study
 263 aimed to identify compounds that could be developed to be administered
 264 alongside front-line treatments with the aim of reducing the rates of recurrent
 265 infection. This may then allow the duration and cost of the treatment to be
 266 reduced.

267

268 For this work, *B. thailandensis* is an ideal model organism. It is a close relative
 269 of the Tier 1 biowarfare agents *B. pseudomallei* and *B. mallei* [41], but does not
 270 typically cause disease. Like other *Burkholderia*, a significant percentage of *B.*
 271 *thailandensis* cells in stationary phase generally survive treatment with
 272 ceftazidime [20], and this organism is resistant to several standard laboratory
 273 antibiotics. It is easy to culture and grows well in standard media. These
 274 properties allowed us to design a robust assay and transfer it between
 275 laboratories without significant re-optimization or institutional barriers.

276

277 We decided that the use of a whole cell, phenotypic assay was advantageous
 278 for this application. Cells in the low metabolic state that provide resistance to
 279 antibiotics such as ceftazidime are heterogeneous [42], and cover a range of
 280 phenotypes. As such, a phenotypic assay focusing on the reductive state of the
 281 cell was preferred over a target based assay for identifying tractable hit
 282 compounds. In addition, phenotypic screening is regaining popularity over
 283 target based screening. The principal reasons for this are that compounds with
 284 the physiological ability to penetrate Gram-negative cells, function *in vivo* and
 285 avoid efflux pumps are identified [43, 44]. Consequently, the compound series
 286 obtained are likely to have significant advantages for downstream optimization
 287 and development. The use of cell viability reagents, allowing the assessment of
 288 cells at a population or individual level, offered the opportunity to identify viable
 289 cells in a variety of states, and so was more relevant for our goals. Resazurin
 290 based assays have previously been shown to identify all viable cells, and not
 291 just the less abundant “persister” cells [45]. Other phenotypic assays that have
 292 identified co-therapies against other organisms have exploited colony counting
 293 [46, 47], DNA binding dyes [48] and Live/Dead reagents [49]. The PrestoBlue
 294 resazurin-based assay proved effective, in *Burkholderia*, at identifying
 295 compounds that were active at concentrations below 10 μ M, validating the
 296 approach. Primary screening with the DDU's diversity library identified 2,127
 297 compounds that showed a significant effect as a co-therapy with ceftazidime,
 298 based on an activity threshold of 34.3% inhibition. The preliminary hit rate was
 299 3.5%, which is in the expected range for an effective assay.

300

301 The assays described here identified chloroxine as a potential a co-therapy to
 302 treat infection with *B. pseudomallei*. This compound demonstrated strong
 303 activity in the primary assay ($IC_{50} = 2 \mu M$), and resulted in a significant
 304 reduction in the number of culturable cells following 24-48 hours treatment in
 305 combination with ceftazidime (>1,000 fold reduction). This is similar to the level
 306 of efficacy that has been previously observed with compounds targeting *E. coli*
 307 [48]. It is hypothesized that chloroxine would reduce the proportion of cells
 308 surviving ceftazidime treatment, and so reduce the intensive treatment phase.
 309 chloroxine, has known bacteriostatic, fungistatic and antiprotozoal properties
 310 [50] and has previously been shown to have synergistic effects with minocycline
 311 against *Pseudomonas aeruginosa* [51]. Consistent with this, administration of
 312 chloroxine alongside the frontline treatment for melioidosis, ceftazidime, had
 313 better activity than either compound evaluated as a sole therapy. This suggests
 314 that the compounds have complementary effects when treating *B.*
 315 *thailandensis*. Bactericidal effects were observed at concentrations below the
 316 chloroxine MIC. This study demonstrates evidence for the concept of use of
 317 chloroxine as a complementary agent to ceftazidime against *B. thailandensis*.

318

319 Hit expansion was carried out for chloroxine. Only a limited range of compounds
 320 around the chloroxine structure were available. None of the compounds
 321 evaluated demonstrated improved activity compared to the parent compound
 322 (Supplementary Table 1). However, it became evident that only limited
 323 substitutions at the chlorine positions were tolerated, only compounds with
 324 other halides in these positions showed comparable activity to chloroxine

325 Furthermore, addition of a methyl group in the 2-position was sufficient to
 326 abolish activity at the concentrations evaluated. This data strongly suggests that
 327 chloroxine has some specificity, and is not a consequence of its suggested
 328 oxidative activity, the addition of a methyl group would not be expected to
 329 reduce the oxidative capability of chloroxine, yet this abolishes activity.
 330 Furthermore, the iodo-equivalent of chloroxine retains similar activity to
 331 chloroxine, and is considerably less oxidizing. This hit expansion validates
 332 chloroxine as a co-therapy for ceftazidime.

333

334 *B. thailandensis* was used in the preliminary experiments as a surrogate for *B.*
 335 *pseudomallei*. Evaluation of chloroxine against *B. pseudomallei* showed that
 336 chloroxine is effective as a co-therapy for ceftazidime at druggable
 337 concentrations. Although the activity is slightly reduced than that observed
 338 against *B. thailandensis*, these results validate the use of *B. thailandensis* as a
 339 surrogate in this study. In the context of ongoing treatment for cutaneous
 340 melioidosis, chloroxine is currently licensed for topical treatment of skin
 341 infections. Because of its low cost and activity against *B. pseudomallei*, it may
 342 become a useful addition to the existing portfolio of (usually effective)
 343 treatments for cutaneous melioidosis. Determining the efficacy against a wider
 344 range of strains of *B. pseudomallei* would provide further confidence in this
 345 concept. Development of co-therapies suitable for systemic treatment would
 346 require significant chemical modification to optimize activity and bioavailability.
 347 A wider range of starting lead scaffolds would likely be necessary for such
 348 optimization.

349

350 In conclusion, our study has demonstrated that a phenotypic assay can identify
 351 compounds that act as co-therapies for frontline antibiotics in *Burkholderia*. A
 352 high throughput screen of 61,250 compounds identified six compounds that
 353 demonstrated activity at concentrations of less than 10 μ M. One of these
 354 compounds, **A**, 5,7-dichloro-8-quinolinol (chloroxine), is currently licensed for
 355 other indications. Although hit expansion with commercially available
 356 compounds did not identify any neighbors with improved activity, chloroxine
 357 significantly reduces the numbers of surviving cells over 48 hours. Our data
 358 suggest that similar approaches could be highly efficacious in identifying useful
 359 compounds for use with other bacteria with similar clinical challenges.

360

361 **Experimental Procedures**

362 **Bacterial strain and culture conditions.**

363 *B. thailandensis* strain E264 (ATCC; strain 700388) was grown overnight in high
 364 salt (10 g/L) lysogeny broth (LB) at 37 °C with aeration at 200 rpm. For
 365 experiments investigating co-antibiotic activity, *B. thailandensis* was grown to
 366 stationary phase in LB broth and cells harvested by centrifugation. Cell pellets
 367 were resuspended in M9 minimal media [52] supplemented with 400 µg/ml
 368 ceftazidime hydrate (Melford Laboratories, #C5920). For growth of *B.*
 369 *pseudomallei* strain K96243 (S. Songsivilai, Siriraj Hospital), bacteria were
 370 plated onto low salt (5 g/L) LB-agar. Single colonies were picked into 100 ml low
 371 salt LB broth and grown at 37 °C for 20 hours with orbital shaking. Cells were
 372 harvested by centrifugation and pellets resuspended in M9 minimal media.
 373 Ceftazidime was prepared from a stock at 10 mg/ml active component in 0.1 M
 374 sodium hydroxide. Chloroxine (Sigma-Aldrich, #D64600) was prepared from a
 375 stock at 10-100 mg/ml active component in dimethyl sulfoxide (DMSO).

376

377 **Cell viability assay**

378 Detection of cell viability with PrestoBlue™ (Life Technologies, #A13261) was
 379 performed in 96 and 384 well, black walled assay plates (Corning, #3904 and
 380 #3573 respectively) by adding 10% PrestoBlue (v/v) to each bacterial culture.
 381 Following the addition of PrestoBlue, plates were incubated at room
 382 temperature for one hour and fluorescence was read at ex 540 / em 590 nm by
 383 an Envision plate reader (PerkinElmer), or an Infinite M200 Pro (Tecan). All
 384 liquid handling in the primary screen and hit expansion was automated.

385 An assay was developed to discriminate two-fold changes in cell numbers. A
386 bacterial culture was prepared as described above and serially diluted in an
387 equal volume of M9 media to produce two-fold dilutions. A positive control (cells
388 resuspended in M9 media without ceftazidime hydrate) and a negative control
389 (cells heat killed at 90 °C for 2 minutes) were included in these assays. Plates
390 were incubated at 37°C overnight before addition of PrestoBlue reagent and the
391 reading of fluorescence as described previously.

392

393 **High throughput screening.**

394 A library of 61,250 compounds was prepared as stock solutions in DMSO at a
395 concentration of 10 mM and supplied in 384-well Echo plates (Labcyte, #P-
396 05525) for use in this screen. 45 µl of a culture resuspended in M9 media
397 supplemented with 400 µg/mL ceftazidime hydrate to an OD_{600 nm} of 0.8
398 (equivalent to late log phase growth, where ceftazidime is expected to be
399 effective) was added to give a final compound concentration of 30 µM. Plates
400 were covered with AeraSeal film (Sigma-Aldrich, #A9224) before incubation for
401 24 hours at 28 °C. A single point (SP) screen of all compounds was performed.
402 309 compounds from the diversity library were tested for potency using a
403 standard ten point half logarithm concentration response protocol [53].
404 Selected hits were dispensed into 384 well Echo plates using the Biomek FX
405 automated liquid handling workstation (Beckman Coulter); two-fold serial
406 dilutions of each compound in DMSO was performed using an Echo 550 liquid
407 handler (Labcyte).

408

409 Our specifications for assay design stipulated a Z factor > 0.5 [54] [36].

410

$$Z\ Factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

411

412 **Data processing and analysis.**

413 Data analysis was performed within ActivityBase (IDBS) and report creation
414 was undertaken using Vortex (Dotmatics). All IC₅₀ curve fitting was undertaken
415 within Activity Base XE utilizing the underlying 'MATH IQ' engine of XLfit version
416 5.1.0.0 from IDBS. Curve fitting was carried out using the following 4 parameter
417 logistic equation: $y = A + (B - A) / (1 + ((10^C) / x)^D)$, where A = % inhibition at bottom,
418 B = % inhibition at top, C = 50 % effect concentration (IC₅₀), D = slope,
419 x = inhibitor concentration and y = % inhibition. As IC₅₀ values are Log normally
420 distributed, fitted IC₅₀ values are stated as the pIC_{50} ($-\log_{10}[IC_{50}]$).

421

422 **Minimum Inhibitory Concentration (MIC).**

423 These were determined following the CLSI recommended protocol for
424 antimicrobial susceptibility testing via micro dilution method [55, 56].
425 Experiments were initiated with an inoculum of $\sim 10^5$ cfu of *B. thailandensis*
426 E264, testing a concentration range from 0-1000 μ M. Growth was detected by
427 absorbance at 600 nm using an Infinity M200 Pro plate reader (Tecan).
428 Synergistic interactions of chloroxine and ceftazidime were tested by mixing
429 equal volumes of media prepared using the micro dilution method, to test

430 concentrations of each antibiotic from 0-32 µg/ml. Samples were then treated
431 as above.

432

433 **IC₅₀ determination**

434 90 µL of a culture prepared as above and resuspended in M9 media
435 supplemented with 400 µg/mL ceftazidime hydrate to an OD_{600 nm} of 0.8 was
436 treated with two-fold dilutions of compounds in DMSO, (in a final DMSO
437 concentration of 0.016% (v/v)). Samples were incubated in a 96 well black
438 walled plate covered with AeraSeal film (SigmaAldrich) at 28 °C for 24 hours
439 before quantification of viable cells with PrestoBlue as above. IC₅₀ values were
440 fitted using Graphpad Prism version 6.0.1.

441

442 **Live/dead screening**

443 A secondary assay was performed to determine the proportion of live/dead cells
444 in response to treatment with two-fold dilutions of the compound. Cultures were
445 prepared and incubated as above. 3 µl of BacLight Live/Dead reagent (Life
446 Technologies, #L7012) was added to each well from an equal volume stock
447 solution of SYTO9 and propidium iodide in DMSO and mixed thoroughly. Plates
448 were incubated at room temperature in the dark for 15 minutes and
449 fluorescence was read at ex 480 / em 500 nm for SYTO9 stain and ex 490 / em
450 635 nm for propidium iodide.

451

452 **Time dependent killing**

453 A stationary phase culture of *B. thailandensis* was centrifuged, resuspended in
 454 10 ml fresh LB media to an OD_{600 nm} of 0.4. Samples were treated with 400
 455 µg/ml ceftazidime hydrate, 30 µM compound **A** in DMSO, or both. Samples
 456 were incubated at 37 °C with shaking. 1 mL samples were taken at time
 457 intervals over a 48 hour period (0, 4, 8, 24, 48 hr), cells harvested and
 458 resuspended in fresh LB media before serial dilution and plating on agar.
 459 Colonies were counted after 24 hours incubation at 37 °C. All samples
 460 contained DMSO at 0.083% (v/v).
 461

462

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658 Figures

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660 **Figure 1: Structures of compounds detailed in this study. A:** 5,7-dichloro-8-quinolinol,
 661 clinically known as chloroxine; **B:** 5-ethyl-5'-(2-hydroxyphenyl)-3'H-spiro[indole-3,2'-
 662 [1,3,4]thiadiazol]-2(1H)-one.

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665 **Figure 2: The PrestoBlue assay shows discrimination between the numbers of surviving**
666 **cells.** A *B. thailandensis* culture was harvested and resuspended in M9 media supplemented
667 with 400 µg/ml ceftazidime hydrate, and diluted in ceftazidime supplemented M9 media to
668 provide a series of cell densities at two-fold intervals. The positive control was prepared in the
669 same media without the addition of ceftazidime; the negative control was a similar sample to the
670 positive control, killed by heating. Samples were incubated statically at 28 °C in 96 well plates.
671 After 20 hours, PrestoBlue was added and the fluorescence read gain optimized for the highest
672 bacterial concentration. The results show reliable discrimination of two fold differences in cell
673 numbers as compared to a heat killed cell negative control. * All initial seeding densities at
674 3.9×10^8 CFU/mL or greater show $Z' > 0.5$ when compared to the negative control and a media
675 only control. Data shows biological triplicates, whiskers indicate min and max results, box 25th to
676 75th percentile, central line indicates median.

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679 **Figure 3: Inhibitory activity of test compounds screened with a phenotypic assay. A B.**

680 *thailandensis* culture was harvested and resuspended in M9 media supplemented with
681 ceftazidime hydrate, to which compounds were added at 30 μ M. Cells were grown at 28 $^{\circ}$ C for
682 20 h, and PrestoBlue added. Inhibition was calculated by comparisons to DMSO controls (0%
683 inhibition) and heat killed controls (100% inhibition). The distribution shows percentage
684 inhibition grouped in 5% windows for high throughput screening of 61,250 compounds. The
685 median activity is 5.3%. The standard deviation of the positive tail is 9.65%, giving a statistical
686 cut-off for activity of 34.3%. The red arrow indicates the selected pragmatic threshold at 45%:
687 345 compounds were identified as 'hits' according to this criterion. 309 compounds were
688 selected based on a high level of activity, or as analogues of compounds with a high level of
689 activity present in the library.

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692 **Figure 4: pIC_{50} determination of two candidate compounds using the PrestoBlue cell**
693 **viability assay.** *A. B. thailandensis* culture was harvested, and resuspended to a concentration
694 of 8×10^8 CFU/mL in M9 media supplemented with 100X MIC ceftazidime hydrate. This was
695 added to a 96 well plate containing two-fold dilutions of compounds in DMSO. Plates were
696 incubated for 24 hours at 37 °C before the addition of PrestoBlue cell viability reagent and the
697 fluorescence read. Results show three biological replicates with error bars indicating standard
698 deviation.

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	Top	Bottom	Hill Slope	pIC_{50}	R Square	Number of Points Analysed
A	37600	10700	1.7	5.5	0.98	42
B	33400	11900	3.8	4.6	0.95	45

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701 **Table 1: Analysis of the concentration dependent killing data shown in Figure 4.**

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704 **Figure 5: The pIC_{50} determination from compound B using the Live / Dead cell viability**
 705 **assay.** The Live / Dead reagents SYTO9 and propidium iodide were used to quantify viability as
 706 a function of the membrane integrity of the cell. A *B. thailandensis* culture was harvested, and
 707 resuspended to a concentration of 8×10^8 CFU/mL in M9 media supplemented with 100X MIC
 708 ceftazidime hydrate. This was added to a 96 well plate containing two-fold dilutions of
 709 compounds in DMSO. Plates were incubated for 24 hours at 37 °C before addition of Live /
 710 Dead cell viability reagents and the fluorescence read. Results show three biological replicates
 711 with error bars indicating standard deviation. Compound B has a pIC_{50} of 4.95 in this assay.

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715 **Figure 6: A secondary assay evaluating the number of culturable cells remaining**
716 **following treatment with ceftazidime and chloroxine.** A culture of *B. thailandensis* was
717 treated with 400 µg/ml ceftazidime hydrate, 30 µM **chloroxine**, both, or neither. Samples were
718 incubated at 37 °C with shaking. Samples were taken at time intervals, cells harvested and
719 resuspended in LB broth before serial dilution and enumerating on agar. Error indicates
720 standard error of serial dilution and CFU count. $n=6$. Differences between the ceftazidime alone,
721 **chloroxine** alone, and ceftazidime and **chloroxine** samples were analyzed using a Kruskal-
722 Wallis test with Dunn post-hoc comparison using Graphpad v. 7.03. * - $p < 0.01$ between
723 **chloroxine** alone, and ceftazidime with **chloroxine**. ** - $p < 0.05$ between both **chloroxine**
724 alone and ceftazidime alone, and both compounds.

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Figure 7: Hit expansion around chloroxine. A *B. thailandensis* culture was harvested, and resuspended to a concentration of 8×10^8 CFU/mL in M9 media supplemented with 100X MIC ceftazidime hydrate. This was added to a 96 well plate containing two-fold dilutions of compounds in DMSO. Plates were incubated for 24 hours at 37 °C before addition of PrestoBlue cell viability reagent and the fluorescence read. Results show three biological replicates with error bars indicating standard deviation. 7-amino-5-chloro-8-quinolinol differs to chloroxine through substitution of an amino group for a chlorine at position 7 (A). This modification causes a significant decrease in this compound's activity as a co-treatment with ceftazidime, with a $pIC_{50} \approx 3.4$. 5,7-dichloro-2-methyl-8-quinolinol differs from chloroxine by addition of a methyl group in the 2-position (B). This addition abolishes this compound's activity as a co-treatment with ceftazidime at the concentrations tested. 7-Bromo-8-quinolinol differs from chloroxine by the removal of chlorine at the 5-position, and replacement of chlorine by bromine at the 7-position (C). This compound retains activity as a co-treatment with ceftazidime that is comparable with the parent compound ($pIC_{50} = 5.1, 5.5$ for chloroxine).

Figure 8: pIC_{50} determination using the PrestoBlue cell viability assay to compare the concentration dependent killing for ceftazidime used in combination with chloroxine to treat *B. thailandensis* and *B. pseudomallei*. A *B. thailandensis* culture was harvested, and

748 resuspended to a concentration of 8×10^8 CFU/mL in M9 media supplemented with 100X MIC
 749 ceftazidime hydrate. This was added to a 96 well plate containing two-fold dilutions of
 750 chloroxine in DMSO. Plates were incubated for 24 hours at 37 °C before the addition of the
 751 PrestoBlue cell viability reagent and determination of fluorescence. Results show three
 752 biological replicates with error bars indicating standard deviation. pIC_{50} for *B. thailandensis* =
 753 5.7; pIC_{50} for *B. pseudomallei* = 5.0.

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