https://dx.doi.org/10.1016/j.micres.2024.128006

Antimicrobial effect of sulconazole in combination with glucose/trehalose against carbapenem-resistant hypervirulent *Klebsiella pneumoniae* persisters

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Abstract

The emergence and rapid dissemination of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) pose a serious threat to public health. Antibiotic treatment failure of *K. pneumoniae* infections has been largely attributed to acquisition of antibiotic resistance and bacterial biofilm caused by the presence of antibiotic persisters. There is an urgent need for novel antimicrobial agents or therapy strategies to manage infections caused by these notorious pathogens. In this study, we screened a collection of compounds that can dissipate bacterial proton motive force (PMF) and intermediate metabolites that can suppress antibiotic tolerance, and identified an antifungal drug sulconazole which can act in combination with glucose or trehalose to exert strong antibacterial effect against starvation-induced CR-hvKP persisters. Investigation of underlying mechanisms showed that sulconazole alone caused dissipation of transmembrane PMF, and sulconazole used in combination with glucose or trehalose could significantly inhibit the efflux activity, reduce NADH and ATP levels, and cause intracellular accumulation of reactive oxygen species (ROS) in CR-hvKP persisters, eventually resulting in bacterial cell death. These findings suggest that the sulconazole and glucose/trehalose combination is highly effective in eradicating multidrug-resistant and hypervirulent *K. pneumoniae* persisters, and may be used in development of a feasible strategy for treatment of chronic and recurrent *K. pneumoniae* infections.

Keywords: Carbapenem-resistant hypervirulent *Klebsiella pneumoniae*, persisters, antimicrobial effect, sulconazole, glucose, trehalose

1. Introduction

Persisters are a subpopulation of susceptible bacteria which can survive lethal dosages of antibiotics because of their inactive physiological state (Lewis, 2010). Unlike antibiotic resistant strains which contain genetic modifications, persisters are genetically identical to the susceptible cells, exhibit the same minimal inhibitory concentration values but a different drug susceptibility phenotype in which they are tolerant to antibiotics at concentrations that would otherwise be lethal (Brauner et al., 2016). Several factors appear to induce the formation of persisters. First, persisters exist independently in the presence of antibiotics and other environmental signals (Helaine and Kugelberg, 2014). In addition, a variety of other environmental factors have been found to trigger persister formation such as starvation (Bernier et al., 2013), carbon source transitions (Amato et al., 2013), quorum sensing (Möker et al., 2010), host macrophages (Helaine et al., 2014) and antibiotics (Dörr et al., 2010). Furthermore, various mechanisms involving the toxin-antitoxin modules (Harms et al., 2016), respiration and energy production (Shan et al., 2017), efflux pumps (Pu et al., 2016) and drug target inactivation (McKay and Portnoy, 2015) can induce persister formation. Eradication of persisters with antibiotics is currently a major challenge since traditional antibiotics are mainly effective against actively growing bacteria only. Strategies to eradicate persisters are mainly categorized into three types, including killing persisters in the dormant state, resensitizing persisters to traditional antimicrobial agents, and avoiding formation of persisters (Wood, 2016). Persisters have been demonstrated to be rapidly activated by glycolysis intermediates and sugars, in which the resultant regrowing cells are susceptible to aminoglycoside (Allison et al., 2011). In addition, treatment with a combination of antibiotics is considered an effective way to eliminate persisters. For instance, polymyxin B

when used in combination with meropenem was recently demonstrated to successfully eradicate persisters from *Acinetobacter baumannii* (Gallo et al., 2017).

To date, persisters are found to be ubiquitous among different bacterial species and have been well investigated in major pathogens such as *Escherichia coli* (Shan et al., 2017), *Staphylococcus aureus* (Conlon et al., 2016) and *Mycobacterium tuberculosis* (Vilchèze et al., 2017). Studies in persisters have been rarely reported in *Klebsiella pneumoniae*, which is one of the important hospital pathogens of increasing clinical concern and is often found in biofilm-related chronic and recurrent infections. Bacterial biofilm is considered as another important cause of therapy failure of *K. pneumoniae* due to the existence of antibiotic tolerant persisters that frequently result in persistent infections (Vuotto et al., 2014). Persisters are responsible for antibiotic tolerance, biofilms and recurrent infections in various pathogens such as *K. pneumoniae*, which generates high levels of tolerant persisters to survive under treatment by a variety of high concentrations of bactericidal antibiotics (Ren et al., 2015; Michiels et al., 2016; Li et al., 2018) and formation of persister is prevalent in clinical *K. pneumoniae* strains (Li et al., 2018). Bacterial population is often exposed to sub-bactericidal concentrations of antibiotics during treatment or in clinical environments, which significantly increased persister formation in *K. pneumoniae* strains (Li et al., 2018), gravely threatening our ability to manage persistence infections. Carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) is a type of *K. pneumoniae* that expresses both carbapenem resistance and hypervirulence phenotypes, causing life-threatening community-acquired and nosocomial infections that cannot be cured by almost all currently available antibiotics. In addition, CR-hvKP strains also express multidrug

tolerance, which is responsible for these biofilm-related chronic *K. pneumoniae* infections. Therefore, development of novel antimicrobial agents or therapy strategies for the eradication of CR-hvKP persisters is increasingly needed. Sulconazole is an imidazole derivative with broad-spectrum antifungal activity and has been commonly used to treat skin fungal infections, including dermatophyte infections, pityriasis versicolor and candidiasis (Benfield and Stephen, 1988). Sulconazole was also found to exhibit antimicrobial activity against the multidrug-resistant *Salmonella typhimurium* (Preethi et al., 2016), in which the antibacterial activity may be due to the inhibition of DNA gyrase and topoisomerase (Tanitame et al., 2004). In this study, we demonstrated that sulconazole used in combination with glucose or trehalose exhibited potent antimicrobial effect against starvation-induced CR-hvKP persisters. The bactericidal effect of sulconazole in combination with glucose or trehalose against CR-hvKP persisters was evaluated and the underlying mechanisms were explored.

2. Materials and methods

2.1. Bacterial strains and drugs

CR-hvKP strain HvKP2 used in this study was isolated from patient in our previous study (Gu et al., 2018). Briefly, CR-hvKP isolate HvKP2 was resistant to carbapenems, cephalosporins, quinolones and aminoglycoside but remained susceptible to polymyxins and tigecycline. ST11 K47 CR-hvKP strain HvKP2 carried a pLVPK-like virulence plasmid conferring the hypervirulence phenotype. Sulconazole nitrate salt (CAS no. 61318-91-0), glucose (CAS no. 50-99-7) and trehalose (CAS no. 6138-23-4) were purchased from Sigma-Aldrich (USA).

2.2. Time-dependent killing assay

To generate a tolerant population (Wang et al., 2022), overnight culture of CR-hvKP isolate was 100-fold diluted into fresh Luria-Bertani (LB) broth and incubated for about 2h to reach the exponential phase. Bacterial culture was then washed twice and resuspended in saline (0.85% NaCl), followed by incubation at 37℃ for 24h to create starvation stress. After starvation for 24h, bacterial culture was treated with sulconazole, glucose, or different combinations at 37℃; bacterial culture resuspended in saline was included as a control group. Viable bacterial counts were determined at 0h, 24h, 48h, 72h, and 96h by serial dilution. Killing curves were depicted by plotting viable counts against incubation time using GraphPad Prism 8 (San Diego, CA).

2.3. Membrane potential assay

The ability of sulconazole, glucose or combinations to induce dissipation of membrane potential in CR-hvKP strain was evaluated using a voltage-sensitive dye, 3,3-dipropyl-thiadicarbocyanine

[DiSC3(5)] (Hamamoto et al., 2015). CR-hvKP strain subjected to 24h starvation was washed and resuspended in PBS supplemented with 0.1M KCl to a final concentration of OD_{600} of 0.2. Bacterial suspension was then incubated with 1μM DiSC3(5) for 15min in the dark, followed by treatment with sulconazole, glucose or different combinations of the two compounds; bacterial suspension without treatment was included as a control group. Valinomycin (1μg/mL) was included as a positive control. Fluorescence intensity was monitored for 10min using a SpectraMax iD3 multimode microplate reader with excitation wavelength at 610nm and emission wavelength at 660nm.

Compounds that dissipated PMF were screened from a drug library using the membrane potential assay as mentioned above. 2μg/mL valinomycin was included as a positive control. A compound caused a fluorescence level similar to or higher than that of 2μg/mL valinomycin was considered as a PMF dissipator. Screening of intermediate metabolites that suppressed starvation-induced antibiotic tolerance was performed by using a phenotype microarray (PM1 - PM5 microplates, Biolog) as previously reported (Wang et al., 2020).

2.4. NADH and ATP measurements

CR-hvKP strain subjected to 24h starvation was then treated with sulconazole, glucose or various combinations at 37℃ for 24h and bacterial culture resuspended in saline was included as a control group, followed by washed and resuspended in cold PBS to a final concentration of OD_{600} of 0.2. The concentration of NADH was determined using an EnzyChrom NAD/NADH Assay Kit (BioAssay Systems). Briefly, bacterial cells (10^8 CFU) were homogenized with 100μL NADH extraction buffer and incubated at 60℃ for 5min. Then, 20μL Assay Buffer and 100μL

opposite extraction buffer were added to neutralize the extracts. Samples were briefly vortexed and centrifuged at 14,000rpm for 5min. Next, 40μL supernatant and 80μL working reagent were quickly added into each well of a clear flat-bottom 96-well plate. Absorbance was measured at 0min and after a 15min incubation at 565nm. NADH concentration was determined from a standard calibration curve depicting the relationship between the concentration of NADH and optical density (OD_{565}). Bacterial culture subjected to 24h starvation was then treated with sulconazole, glucose or different combinations for 30min and bacterial culture resuspended in saline was included as a control group, intracellular ATP level was determined with an ATP Determination Kit (Invitrogen). Results were presented as mean and standard deviation of data of three independent experiments.

2.5. Assessment of efflux inhibitory activity

The effect of sulconazole, glucose and different combinations on efflux activities in strain HvKP2 was assessed with a dye Nile Red as previously described (Reens et al., 2018). Briefly, bacterial culture subjected to 24h starvation was treated with sulconazole, glucose or different combinations for 24h, and bacterial culture resuspended in saline was included as a control group. Then, bacterial culture was adjusted to OD_{600} at 0.2 and stained with 5μM Nile Red for 3 hours at 37°C. After staining, bacterial culture was washed and resuspended in PBS supplemented with 1mM $MgCl_2$. Fluorescence intensity was monitored for 3min using 96-well black plate with excitation wavelength at 552nm and emission wavelength at 636nm, after which suspension was triggered with 50mM glucose and fluorescence intensity was then monitored for another 10min. The addition of 50mM glucose was used to energize the efflux pump and trigger the efflux of

Nile Red. After treatment with sulconazole, glucose or different combinations for 24h, followed by Nile Red staining and addition of 50mM glucose, bacterial culture was observed with a Nikon Eclipse Ti2 Microscope (Nikon, Japan) and fluorescence images were captured.

2.6. Intracellular ROS accumulation

To assess the level of intracellular ROS accumulation in HvKP2 persisters upon treatment with sulconazole, glucose and different combinations, DCF-DA assay was conducted as previously described (Dridi et al., 2015). Briefly, bacterial cultures subjected to 24h starvation were challenged with sulconazole, glucose or different combinations for 4h, and bacterial culture resuspended in saline was included as a control group. Bacterial cultures were then washed once and resuspended with warm PBS, followed by staining with 10μM DCF-DA for 30min. After staining, cultures were washed and resuspended with warm PBS. Fluorescence intensity was determined with excitation wavelength at 485nm and emission wavelength at 535nm. Viable counts in bacterial culture were determined by plating serial dilutions on LB agar. The level of intracellular ROS accumulation was depicted as fluorescence intensity normalized to viable bacterial counts.

2.7. RNA sequencing and differential expression analysis

Bacterial culture subjected to 24h starvation was then treated with 10μg/mL sulconazole, 2mM glucose, or combinations of 10μg/mL sulconazole and 2mM glucose at 37℃ for 24h, and bacterial culture resuspended in saline was included as a control group. Total RNA was extracted by RNeasy Protect Bacteria Mini Kit (Qiagen, Germany), followed by DNase treatment using

Turbo DNA free kit (Invitrogen). RNA samples were then sent to Novogene Bioinformatic Technology for transcriptome sequencing. Genome of HvKP2 was used as the reference and genes were annotated by eggNOG-mapper (Cantalapiedra et al., 2021). Reads were mapped to the reference genome by Hisat2 (Kim et al., 2019). Quantification process was performed with featureCounts (Liao et al., 2014). A highly variable gene was defined as one of the top 500 variable genes between conditions with |log2 fold change| >1.5. Gene ontology (GO) analysis was used to classify the high variable genes with clusterProfile (Wu et al., 2021). RNA sequencing data has been deposited into the NCBI database under the accession number PRJNA1065810.

2.8. Statistical analyses

Statistical analyses were carried out by unpaired students' *t* test. Data was processed and presented by GraphPad Prism 8.

3. Results

3.1. Screening of specific compounds that dissipated PMF and intermediate metabolites that suppressed starvation-induced antibiotic tolerance

We set out to identify specific PMF dissipators from a drug library through a fluorescence-based high-throughput screening using a dye DiSC3(5), and identify specific intermediate metabolites that could suppress starvation-induced antibiotic tolerance in the presence of antibiotic through a phenotype microarray screening (PM1-PM5 microplates). Particularly, the screening identified 11 compounds (including benzethonium chloride, cetylpyridinium chloride, alexidine HCl, econazole, miconazole nitrate, sulconazole nitrate, dronedarone HCl, clomifene citrate, otilonium bromide, cyclosporine and carbonyl cyanide m-chlorophenylhydrazone (CCCP)) that specifically dissipated bacterial PMF and 6 intermediate metabolites (including glucose, fructose, trehalose, mannitose, maltose and maltotriose) that could specifically suppress starvation-induced antibiotic tolerance. We then combined the two classes of compounds to test their antimicrobial effects against starvation-induced tolerant CR-hvKP population and demonstrated that sulconazole, when used in combination with glucose or trehalose, exerted strong antibacterial effect to eradicate CR-hvKP persisters (Supplementary Fig S1).

3.2. Sulconazole in combination with glucose/trehalose kills starvation-induced CR-hvKP persisters

To evaluate the antimicrobial effect of the sulconazole and glucose/trehalose combination, time-dependent killing assays were conducted on starvation-induced CR-hvKP persisters. Treatment with 10μg/mL sulconazole alone for 96h could not effectively eradicate the tolerant HvKP2

219 population, while treatment with 4mM glucose alone caused a slight reduction in the bacterial
220 population size. However, upon combined treatment of 5μg/mL sulconazole and 4mM glucose,
221 HvKP2 persisters were completely eradicated at 96h (Fig 1a). Complete eradication of HvKP2
222 persisters was also achieved at 72h upon treatment with a combination of 10μg/mL sulconazole
223 and 4mM glucose (Fig 1b). Similar bactericidal effect was also observed upon treatment with a
224 sulconazole/trehalose combination (Fig 1c, 1d), in which complete eradication of tolerant
225 HvKP2 population can be achieved at 72h upon combined treatment with 10μg/mL sulconazole
226 and 1mM trehalose, and even at 48h upon treatment with 10μg/mL sulconazole in combination
227 with 4mM trehalose (Fig 1d). These results suggested that sulconazole, when used in
228 combination with glucose or trehalose, exhibited strong bactericidal effect against starvation-
229 induced CR-hvKP persisters. Sulconazole was also found to exhibit more effective killing
230 against starvation-induced HvKP2 persisters than meropenem (40μg/mL) when in combination
231 with 1mM glucose/trehalose (Supplementary Fig S2 a, b). In addition, we further evaluated the
232 antimicrobial effect of sulconazole in combination with glucose against more clinical pathogens
233 including carbapenem-resistant *K. pneumoniae* strains EH62 and HKU20 (Supplementary Fig
234 S2 c, d), carbapenem-resistant *Escherichia coli* CL4 (Supplementary Fig S2 e) and carbapenem-
235 resistant *Acinetobacter baumannii* CPC35 (Supplementary Fig S2 f) and got similar results.
236
237 **3.3. Sulconazole and glucose/trehalose combination caused dissipation of membrane PMF**
238 Treatment with sulconazole caused rapid dissipation of membrane potential in HvKP2 persisters
239 in a dose-dependent manner with a level much higher than that caused by valinomycin (Fig 2a),
240 while glucose and trehalose could not cause dissipation of membrane potential (Fig 2b, 2c).

241 However, combined treatment of 10μg/mL sulconazole with glucose caused a slight increase in
242 membrane potential, which was similar to the level caused by 1μg/mL valinomycin but lower
243 than that recorded for treatment of 10μg/mL sulconazole alone (Fig 2d). Combined treatment of
244 10μg/mL sulconazole with trehalose caused a relatively high level of membrane potential
245 dissipation when compared to treatment with 10μg/mL sulconazole and glucose combination,
246 the level of which was similar to that recorded during treatment with 10μg/mL sulconazole alone
247 (Fig 2e). The membrane PMF dissipation caused by the drug combination was not as obvious as
248 that caused by sulconazole alone, which may be because the killing effect of glucose on persisters
249 is through different mechanisms from PMF dissipation. The addition of glucose may provide
250 additional energy sources for the bacteria to generate more proton through increased metabolic
251 activities, which in turn render the PMF dissipation effect caused by sulconazole less obvious.
252 Further experiments including metabolic activity relating assays are needed to verify this
253 hypothesis.
254
255 **3.4. Sulconazole and glucose/trehalose combination caused inhibition of efflux activity**
256 Nile red efflux test was performed to investigate the effect of the sulconazole and
257 glucose/trehalose combination on the efflux activities in starvation-induced HvKP2 persisters.
258 Nile red is an efflux pump substrate which would become strongly fluorescent upon partitioning
259 into the bacterial membrane and could be immediately pumped out. Treatment with 10μg/mL
260 sulconazole alone caused moderate decrease in export of Nile red, while treatment of 10μg/mL
261 sulconazole in combination with 4mM glucose resulted in significant reduction in export of Nile
262 red (Fig 3a, 3c). Inhibition of efflux activities was also observed when starvation-induced HvKP2

263 persisters were challenged with the sulconazole and trehalose combination (Fig 3b, 3d).

264

265 **3.5. Sulconazole and glucose/trehalose combination reduced in intracellular NADH and**
266 **ATP level**

267 ATP and NADH play important roles in the growth and survival of living organisms in diverse
268 environments (Hara and Kondo, 2015; Bilan and Belousov, 2016). The generation of ATP is also
269 the most relevant process to membrane potential, which depends on the PMF generated by
270 electron transport chain (Hards and Cook, 2018; Xia et al., 2021). Treatment with sulconazole in
271 combination with glucose or trehalose resulted in a significant decrease in intracellular NADH
272 level when compared to untreated group and these treated with sulconazole alone (Fig 4a, 4b).
273 In addition, intracellular ATP level also significantly decreased upon exposure to the sulconazole
274 and glucose/trehalose combination (Fig 4c, 4d), suggesting that treatment with sulconazole in
275 combination with glucose or trehalose resulted in reduction in the intracellular NADH level and
276 the ability to synthesize ATP, thereby affecting energy supply in HvKP2 persisters.

277

278 **3.6. Sulconazole and glucose/trehalose combination led to intracellular ROS accumulation**

279 Accumulation of ROS in bacteria can trigger growth arrest or cell death through damaging DNA,
280 RNA, proteins and membrane lipids (Wang et al., 2010). Destruction of membrane homeostasis
281 is always due to ROS accumulation which causes increase in membrane permeability and
282 damages when the level exceeds the antioxidant limit of cellular systems (Yang et al., 2022). A
283 previous study reported that PMF dissipation was also associated with overproduction of ROS
284 (Deng et al., 2020). Intracellular ROS level in HvKP2 persisters was determined and results

demonstrated that slightly higher level of ROS accumulation in HvKP2 persisters was induced by combined treatment of sulconazole and glucose/trehalose when compared to sulconazole, glucose or trehalose alone (Fig 4e, 4f), indicating that sulconazole, when used in combination with glucose or trehalose, could potentiate the oxidative damage of bacteria caused by ROS accumulation.

3.7. Sulconazole and glucose combination led to changes of metabolism in CR-hvKP persisters

To further investigate the mechanisms underlying the bactericidal effect of the sulconazole and glucose combination, transcriptome analyses were performed on HvKP2 persisters upon exposure to sulconazole and glucose alone or in combination. GO enrichment analysis within the biological process showed that these differentially expressed genes recorded upon comparison between treatment with sulconazole in combination with glucose and the untreated control include those involved in carboxylic acid catabolism and energy production by oxidation and cellular respiration (Fig 5a). When HvKP2 persisters were exposed to sulconazole in combination with glucose, genes associated with oxidative phosphorylation including those encoding ATP synthase, NADH-quinone oxidoreductase and cytochrome oxidase were remarkably downregulated (Fig 5b). As mentioned, inhibition of the oxidative phosphorylation process and electron transfer chain activities is always due to intracellular ROS accumulation, which may result in bacterial cell death. In this work, genes involved in tricarboxylic acid (TCA) cycle, glycolysis and fatty acid oxidation were also found to be significantly downregulated (Fig 5b), indicating that substantial changes in bacterial metabolic levels occurred. A subset of

differently expressed genes involved in ATP synthesis, NADH production and TCA cycle will be selected for further validation using quantitative real-time PCR.

4. Discussion

Persisters are rare phenotypic variants that exist within a susceptible isogenic subpopulation in all bacterial species and are able to tolerate lethal dosage of antibiotics (Lewis, 2007). Antibiotic tolerance is currently a significant healthcare issue, as persisters constitute an important reservoir of cells that may subsequently emerge as drug-resistant mutants (Barrett et al., 2019; Windels et al., 2019) and cause biofilm-related recurrent infections (Lewis, 2007). Mechanisms underlying persisters formation, survival and recovery are complicated. Stochastic and deterministic mechanisms involved in different cellular processes can cause bacterial growth arrest; these processes, including activities of toxin/antitoxin modules (Schumacher et al., 2009) and stringent response (Amato et al., 2013), may lead to tolerance formation and hence emergence of persisters. Although bacterial growth arrest and dormancy are the most common mechanisms that mediate persister formation, various lines of evidence suggest that persisters may also exhibit diverse physiological activities including those of the active electron transport chains (Orman and Brynildsen, 2015), ATP synthesis (Radzikowski et al., 2016), and synthesis and degradation of RNAs (Mok et al., 2015). Persisters have been described in *K. pneumoniae* in recent years (Ren et al., 2015; Michiels et al., 2016; Li et al., 2018). Given that *K. pneumoniae* is a major source of bacterial antibiotic resistance-encoding genetic elements, and that antibiotic tolerance due to the presence of persisters is closely associated with development of antibiotic resistance (Cohen et al., 2013), further study into mechanisms underlying persister formation in this bacterial species is necessary. In addition, recalcitrant infections related to *K. pneumoniae* may also be attributed to the fact that persisters escape or tolerate the host immunity. Therefore, complete elimination of *K. pneumoniae* persisters needs to be achieved to prevent the chronic and recurrent

K. pneumoniae infections.

Cell membrane is an indispensable component for bacteria and a promising target for development of new antimicrobial therapeutics (Farha et al., 2013). Bacterial PMF is the electrochemical proton gradient across the membrane, which plays an important role in ATP synthesis (Farha et al., 2013). PMF is crucial for bacterial growth and survival under normal and stress conditions (Strahl and Hamoen, 2010). As the driving force for ATP biosynthesis, PMF supplies the necessary energy for numerous cellular processes; therefore, the dissipation of one or both of its components (electric potential $\Delta\psi$ or transmembrane proton gradient ΔpH) will dismantle the cellular adenylate-based energy production system, resulting in bacterial cell death (Farha et al., 2013). However, PMF has been largely disregarded as a target for antimicrobial agents due to the concern of toxicity of PMF dissipators. Previous studies have demonstrated the potency of PMF inhibitors to eradicate bacterial persisters. Dissipating the PMF might be a promising antibacterial strategy against different bacterial species. Compounds that dissipated the PMF inhibited flagellar motility (Paul et al., 2008), which prevented invasion and swarming activity that finally result in biofilm formation (Nan et al., 2011). In addition, PMF-targeted compounds could effectively eradicate dormant bacteria responsible for persistent infections (Lewis, 2007). Dormant bacteria which already exhibit diminished cellular metabolism and a relatively low level of PMF (Hurdle et al., 2011) could therefore be more susceptible to PMF inhibitors. Thus, dissipators of PMF could find their utility as novel therapy strategies against these persistent infections. The compound sulconazole identified in this study can cause dissipation of transmembrane PMF in CR-hvKP persisters, which will become a promising

354 therapy option.

355

356 Metabolites have been previously reported to resensitize bacterial populations to different
357 antibiotics through diverse mechanisms (Allison et al., 2011; Prax et al., 2016; Gutierrez et al.,
358 2017). For instance, daptomycin was reported to exhibit enhanced bactericidal efficiency against
359 *Staphylococcus aureus* persisters in the presence of glucose (Prax et al., 2016). Addition of
360 glucose can trigger the metabolism in *Vibrio cholerae* persisters through generation of PMF and
361 transforming their physiology to the growth status, thereby enhancing their susceptibility to
362 ciprofloxacin (Paranjape and Shashidhar, 2020). Mannitol has been reported to resensitize
363 *Pseudomonas aeruginosa* biofilm to aminoglycosides (Barraud et al., 2013). In addition,
364 metabolites can sensitize the persister cells of *Escherichia coli*, *S. aureus* and *Mycobacterium*
365 *tuberculosis* to quinolones through stimulation of carbon and respiration metabolism (Gutierrez
366 et al., 2017). Specific metabolites have been demonstrated to be associated with varying degrees
367 of killing of tolerant sub-population by stimulating PMF and increasing antibiotic uptake
368 (Allison et al., 2011). The combination of sulconazole and glucose used in this study can
369 eradicate persistent CR-hvKP strains through inhibition of efflux activity, suppression of ATP
370 generation and accumulation of ROS in bacterial cells, resulting in bacterial death. Facilitating
371 TCA cycle by glucose activation that subsequently enhanced PMF-dependent uptake of
372 antibiotic also resulted in higher rate of bacterial cell death in multidrug-resistant *Edwardisiella*
373 *tarda* (Peng et al., 2015). However, transcriptome analysis showed that the combination of
374 sulconazole and glucose caused significant downregulation of genes involved in TCA cycle,
375 glycolysis and fatty acid oxidation, resulting in substantial changes in bacterial metabolic levels.

In this study, we identified several compounds that can dissipate the bacterial PMF, and intermediate metabolites that can suppress the antibiotic tolerance in the presence of antibiotic through high-throughput screenings of an FDA-approved drug library and the testing in Phenotype Microarrays. We demonstrated that the antifungal drug sulconazole could effectively eradicate starvation-induced tolerant CR-hvKP population when used in combination with glucose or trehalose. Investigation of the mechanisms underlying the bactericidal effect of sulconazole showed that this compound caused dissipation of transmembrane PMF, and when used in combination with glucose could significantly inhibit efflux activity, suppress intracellular NADH and ATP production, and cause intracellular accumulation of ROS in CR-hvKP strain, eventually resulting in bacterial cell death. The drug combination seems to affect a wide range of crucial membrane-bound metabolic processes, leading to a downregulation of ATP production, central carbon metabolism and transcription activities. This study has some limitations. The antimicrobial effect of sulconazole in combination with glucose/trehalose was only evaluated in limited bacterial strains, which should be tested in more bacterial strains and bacterial species to expand the application of this drug combination. Mechanisms underlying the bactericidal effect of these compounds need to be further explored, especially these relating to metabolic activity.

5. Conclusions

Here we assessed the antimicrobial effects of sulconazole when used in combination with glucose or trehalose against starvation-induced CR-hvKP persisters and explored the mechanisms underlying the bactericidal activity of these compounds. Sulconazole caused

dissipation of transmembrane PMF, and sulconazole used in combination with glucose significantly inhibited the efflux activity, reduced NADH and ATP levels, and caused intracellular ROS accumulation in CR-hvKP persisters, eventually resulting in bacterial cell death. Sulconazole is a Food and Drug Administration-approved broad-spectrum imidazole antifungal agent. Drug repositioning, the new application of existing clinically approved drugs, can significantly reduce the time and costs of the drug discovery and development process. This study effectively repositions sulconazole as an antibacterial candidate and provides strong experimental evidence for the clinical application of sulconazole in CR-hvKP infections.

Funding

This work was supported by Theme-based Research Scheme (grant number: T11-104/22-R) and General Research Fund (grant numbers: 11100321, 11100922) of Research Grant Council of Hong Kong SAR.

Declaration of Competing Interest

The authors declare no competing interests.

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Figures

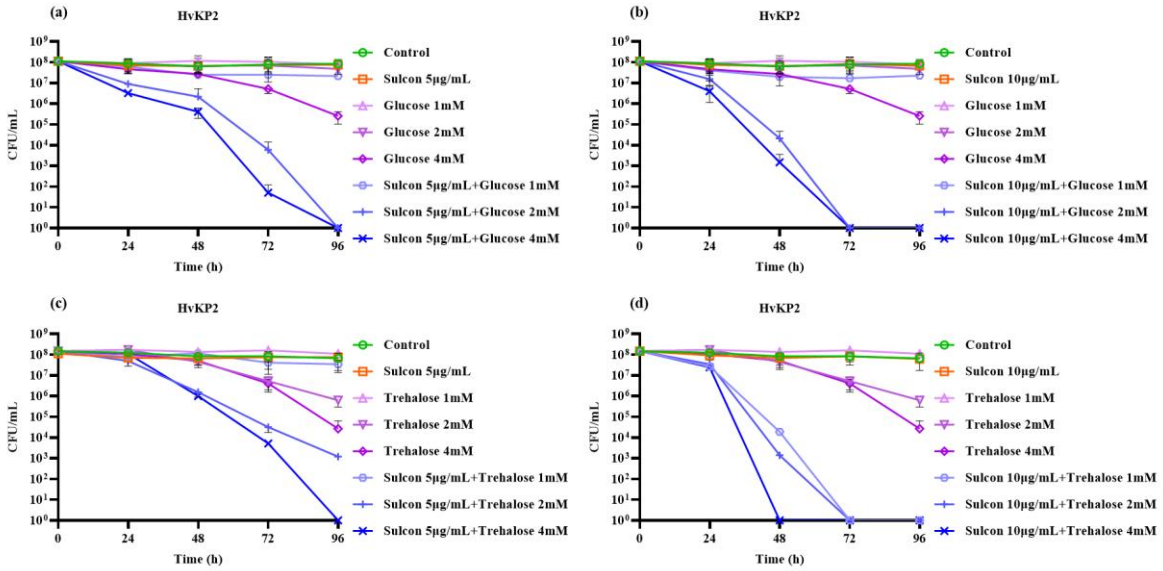

Fig 1. Time-dependent killing assay of CR-hvKP strain HvKP2. (a) and (b) CR-hvKP strain HvKP2 subjected to 24h starvation was treated with sulconazole, glucose or various combinations of both compounds for 96h. (c) and (d) CR-hvKP strain HvKP2 subjected to 24h starvation was treated with sulconazole, trehalose or various combinations of both compounds for 96h. Results were presented as mean and SD of three independent experiments (Some error bars cannot be observed due to the scale of the Y axis, which compress the error bars to the point where they were not visible.). Sulcon, sulconazole.

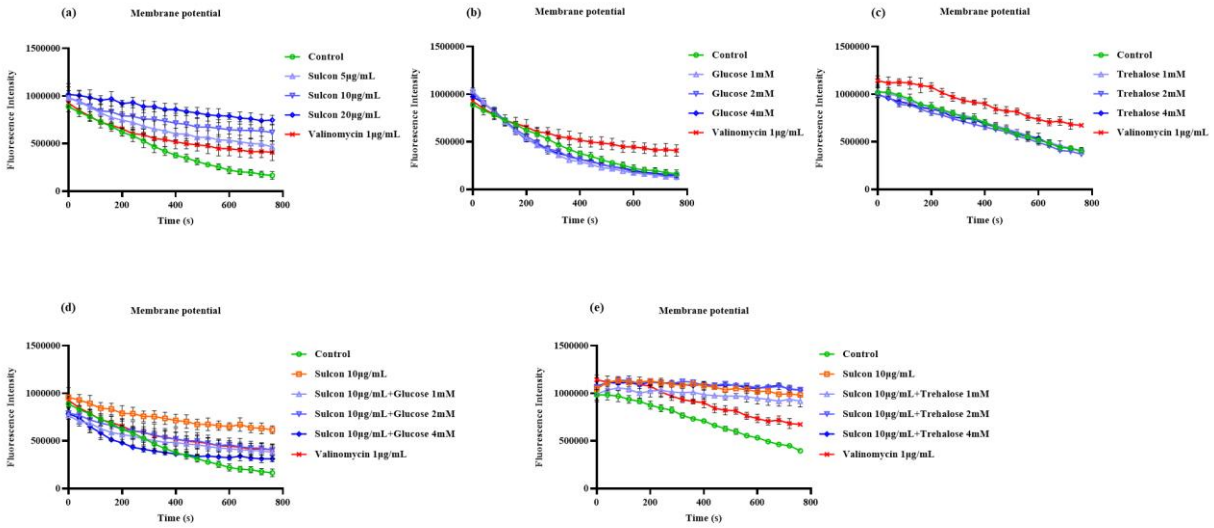

Fig 2. Assessment of membrane potential in CR-hvKP strain HvKP2. Membrane potential was determined in starvation-induced HvKP2 persisters upon treatment with sulconazole (a), glucose (b), trehalose (c), sulconazole in combination with glucose (d), and sulconazole in combination with trehalose (e). Fluorescent dye DiSC3(5) was used to determine the membrane potential, and valinomycin was included as a positive control. Results were presented as mean and SD of three independent experiments.

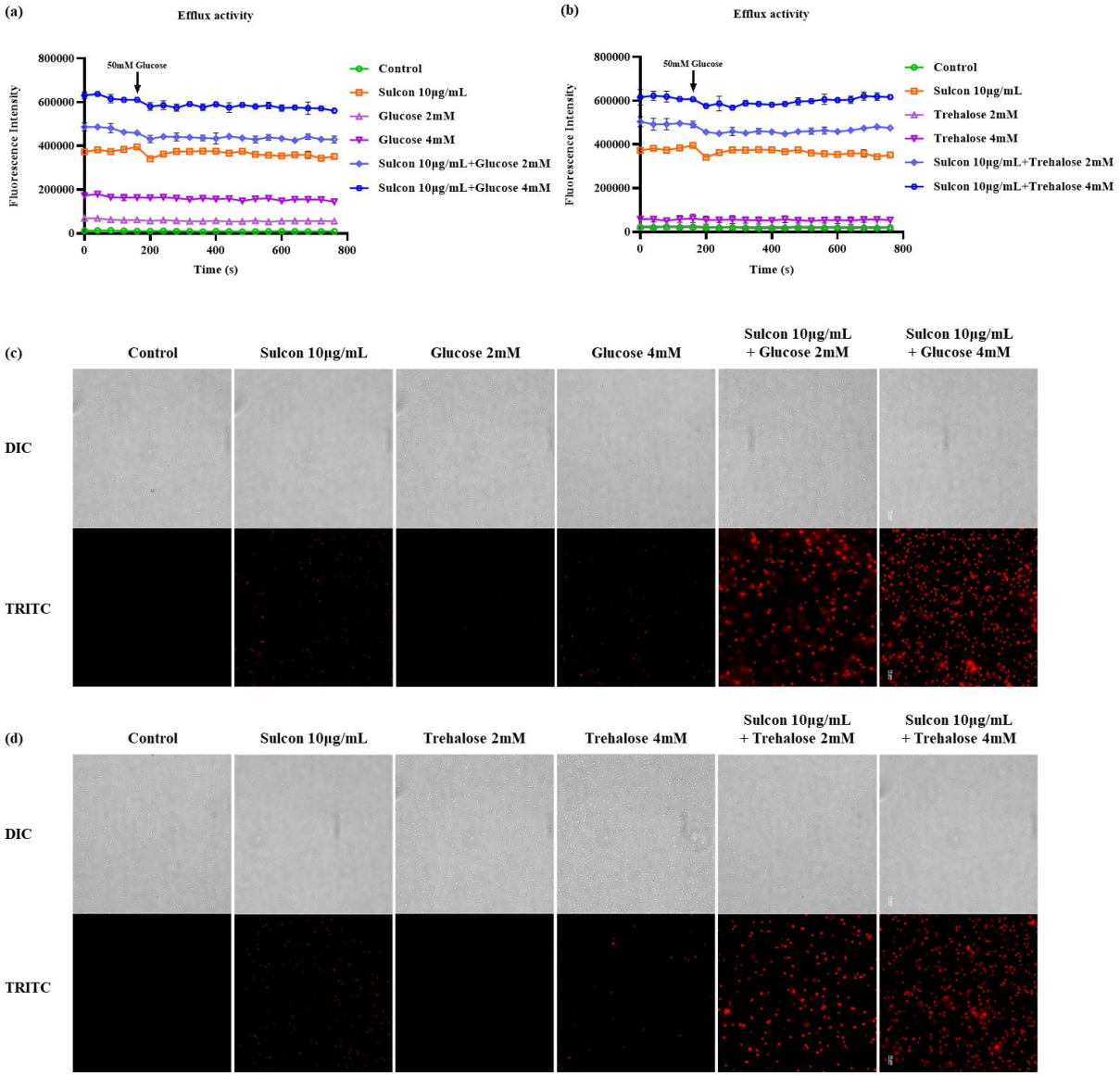

Fig 3. Inhibitory effect of sulconazole in combination with glucose or trehalose on efflux activity in CR-hvKP persisters. Fluorescence intensity of Nile Red in HvKP2 upon treatment with the sulconazole and glucose combination (a), and sulconazole in combination with trehalose (b). Glucose (50mM) was added to bacterial suspension at 3min, and fluorescence intensity was monitored for another 10min. (c) Microscopic images of HvKP2 persisters treated with sulconazole, glucose or combinations, followed by Nile Red staining for 3h. (d) Microscopic images of HvKP2 persisters treated with sulconazole, trehalose or different combinations, followed by Nile Red staining for 3h. Results were presented as mean and SD of three independent experiments (Some error bars cannot be observed due to the scale of the Y axis, which compress the error bars to the point where they were not visible.)

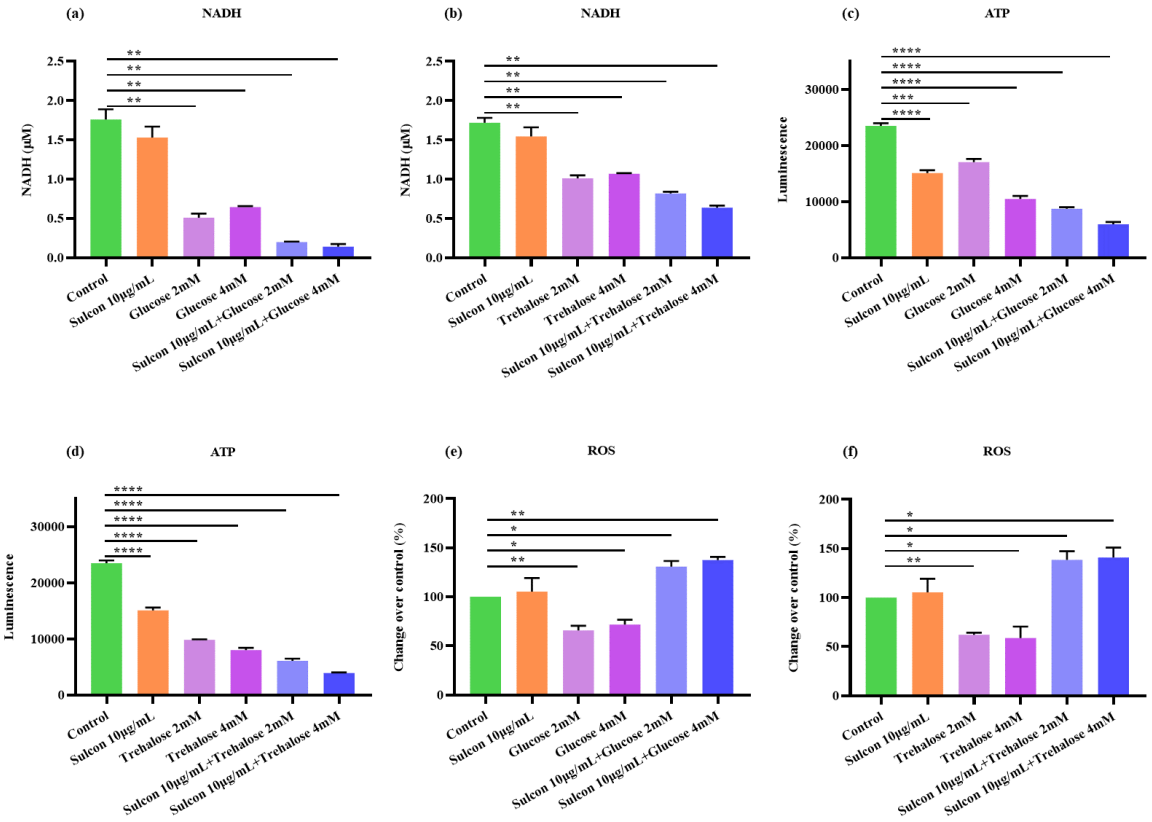

Fig 4. NADH, ATP and ROS production in CR-hvKP persisters. Intracellular NADH level was measured in HvKP2 persisters upon treatment with the sulconazole and glucose combination (a), and sulconazole in combination with trehalose (b). **, 0.001<*P*<0.01. Intracellular ATP levels were measured upon treatment of sulconazole in combination with glucose (c), and sulconazole in combination with trehalose (d) for 30min. ***, 0.0001<*P*<0.001; ****, *P*<0.0001. ROS accumulation in HvKP2 persisters upon treatment of sulconazole in combination with glucose (e), and sulconazole in combination with trehalose (f). Intracellular ROS accumulation was depicted as the fluorescence intensity of ROS normalized to viable cell counts. *, 0.01<*P*<0.05; **, 0.001<*P*<0.01. Results were presented as mean and standard deviation of three tests.

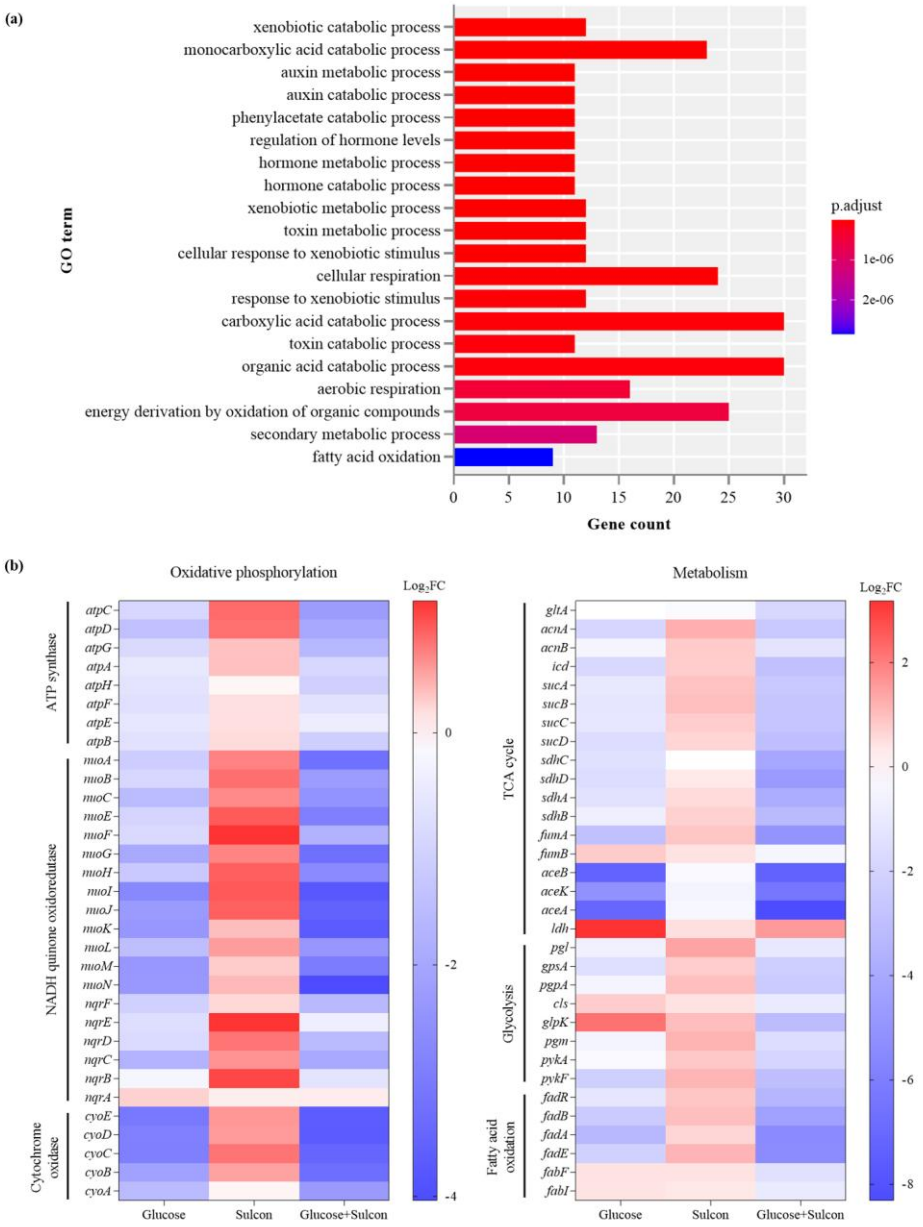

Fig 5. Transcriptome analysis of HvKP2 persisters upon exposure to sulconazole or glucose alone and in combination. (a) Gene Ontology (GO) enrichment analysis. Genes were annotated in biological process, and the top 20 most enriched GO terms between treatment with the sulconazole and glucose combination, and the untreated control, are shown. (b) Effect of glucose, sulconazole alone or in combination on expression of genes involved in oxidative phosphorylation and metabolism. Log_2FC, Log_2 Fold Change.