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Eukaryotic Phosphatase Inhibitors Enhance Colistin Efficacy in Gram-negative Bacteria.

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Abstract

The mounting threat of multi-drug resistant (MDR) bacteria places a tremendous strain on the antimicrobial clinical arsenal, forcing physicians to revert to near-obsolete antibiotics to treat otherwise intractable infections. Antibiotic adjuvant therapy has emerged as a viable alternative to the development of novel antimicrobial agents. This method uses combinations of an existing antibiotic and a non-antimicrobial small molecule, where the combination either breaks drug resistance or further potentiates antibiotic activity. Through a high-content screen of eukaryotic kinase inhibitors, our group previously identified two highly potent adjuvants that synergize with colistin, a cyclic, polycationic antimicrobial peptide that serves as a drug of last-resort for the treatment of MDR Gram-negative bacterial infections. Cell signaling proteins implicated in colistin resistance mechanisms display both kinase and phosphatase activities. Herein, we explore the potential for eukaryotic phosphatase inhibitors to be repurposed as colistin adjuvants. From a panel of 48 unique structures, we discovered that the natural product kuwanon G breaks colistin resistance, while the non-antimicrobial macrolide ascomycin potentiates colistin in polymyxin-susceptible bacteria.

Graphical Abstract

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Conflict of Interest Statement. Dr. C. Melander is co-founder of Agile Sciences, a biotechnology company seeking to commercialize antibiotic adjuvants.

Data Sharing. The data that supports the findings of this study are available in the supplementary material of this article.

1. Introduction

Multiple factors have contributed to the ever-growing litany of problems associated with the treatment of antibiotic resistant bacterial infections. At present, the number of viable options for the treatment of multi-drug resistant (MDR) infections is dwindling as an increasing number of clinical isolates have shown resistance to nearly all first line treatments.(Aslam et al., 2018; Zaman et al., 2017) Further, many reports have documented pan-drug resistant (PDR) pathogens that cannot be treated with any clinically available antimicrobial.(Chen, Todd, Kiehlbauch, Walters, & Kallen, 2017) The cohort of bacterial species that most often develop MDR and PDR characteristics and contribute to the majority of nosocomial infections are known as the *ESKAPE* pathogens, consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.(Mulani, Kamble, Kumkar, Tawre, & Pardesi, 2019) It is estimated that 700,000 lives are currently lost each year to antibiotic resistant infections, a number that is predicted to reach 10 million by 2050.(O'Neill, 2014)

An obvious solution to combat antimicrobial resistance is developing completely new classes of antibiotics, however, in the last three decades, only two new antibiotic classes have been clinically deployed. Lipopeptides (*e.g.* daptomycin) and oxazolidinones (*e.g.* linezolid) both are limited in efficacy to Gram-positive bacteria, and resistance to both was identified shortly after their clinical deployment.(Gonzalez-Ruiz, Seaton, & Hamed, 2016; Stefani, Bongiorno, Mongelli, & Campanile, 2010) With no new treatments available for MDR Gram-negative infections, clinicians are forced to revert to antiquated therapies.

Colistin (polymyxin E) is a polycationic, cyclic polypeptide that was first implemented in the 1950's against Gram-negative infections.(Lim et al., 2010) While highly effective, it carries significant liabilities such as neuro and nephrotoxicity. It now belongs to the "Reserve Group" of antibiotics, as defined by the World Health Organization, and is used only as an antibiotic of last-resort for Gram-negative bacterial infections that do not respond to any other antimicrobial treatment.(Hsia et al., 2019) Bacterial resistance to colistin is also known, warranting a serious effort to develop solutions to preserve its activity. Antibiotic

adjuvant methodology can be employed to identify drug combinations that reverse polymyxin resistance and significantly reduce therapeutic doses.(Barker, Chandler, Melander, Ernst, & Melander, 2019; Harris et al., 2014; Harris, Worthington, & Melander, 2012; Huggins et al., 2018; Rogers, Bero, & Melander, 2010)

Colistin carries a net-positive charge and destabilizes the bacterial cell envelope through electrostatic interactions with the net-negatively charged bacterial cell surface. Bacteria acquire colistin resistance either through point mutations in their endogenous genetic material or by acquiring plasmid-borne genetic elements such as the *mobile colistin resistance* gene family (*mcr* 1-10).(Gelbíová et al., 2019; Hu, Liu, Lin, Gao, & Zhu, 2016; Y.-Y. Liu et al., 2016) Either case results in the upregulation of transferases that covalently modify phosphate moieties on lipid A, a component of lipopolysaccharide (LPS), that decorates the outer cell surface of Gram-negative bacteria with cationic residues.(Pelletier et al., 2013) This reduces the negative charge of the bacterial membrane, which decreases its affinity for colistin and thus reduces its effectiveness.

To date, strains displaying the highest known levels of colistin resistance have been isolated from intensive care facilities where selection pressure gives rise to mutations in two-component systems, such as the *pmrCAB* operon in *A. baumannii*.(Beceiro et al., 2011) The canonical two component system consists of a cell-membrane bound histidine kinase (HK) that auto-phosphorylates upon changes in the extracellular environment and then passes that phosphate group to a response regulator (RR). The phosphorylated RR then has high affinity to the promoter regions of the genes necessary to respond to the detected extracellular change.(Jacob-Dubuisson, Mechaly, Betton, & Antoine, 2018) In the context of colistin resistance, point mutations in the HK gene *pmrB* leads to constitutive activation of this protein and extensive modification of lipid A as a consequence.

Most known HKs are bifunctional and have both kinase and phosphatase activity. The phosphatase activity of the HK is important for regulating the activation of the intended RR while limiting crosstalk with other TCS. The highly labile acyl phospho-Asp bond is easily cleaved and is amenable to dephosphorylation by the HK or an accessory phosphatase under the appropriate stimulus.(Gao & Stock, 2009) We have previously demonstrated that eukaryotic kinase inhibitors can potentiate the activity of colistin against both colistin-resistant and colistin-sensitive bacteria.(Barker, Nemeth, et al., 2019) Based upon this study, we posited that a phosphatase inhibitor could potentially trigger cell-signaling dysregulation and render bacteria more susceptible to antimicrobials. To expound on this inquiry, we have probed the potential for known eukaryotic phosphatase inhibitors to interfere with prokaryotic cell signaling and potentiate colistin. A total of 48 unique structures from two commercially available libraries were screened for activity against both colistin-resistant and colistin-sensitive bacteria. As was the case for our analogous kinase inhibitor study, two compounds emerged with significant but divergent activity. We report reversion of colistin resistance by kuwanon G, a natural product isolated from the root bark of *Morus alba* (white mulberry). This compound has known anti-inflammatory properties and antimicrobial activity against *Streptococcus mutans* and other Gram-positive oral flora.(Park, You, Lee, Baek, & Hwang, 2003) We also report that the natural product ascomycin, a non-antimicrobial macrolide with strong immunosuppressant properties, potentiates colistin in

polymyxin-susceptible bacteria.(Wang, Wang, Song, & Wen, 2017) We show that both of these compounds have activity in a number of Gram-negative strains, and do not act by causing a significant increase in cell permeability. Furthermore, we demonstrate that these products do not have inherent antimicrobial effects at their active adjuvant concentrations in Gram-negative bacilli and offer two new molecular platforms for the potential therapeutic development of colistin adjuvants.

2. Results and Discussion

2.1 Phosphatase Inhibitor Library Screening

A major challenge beset on this study was the paucity of well-characterized and commercially available phosphatase inhibitors compared to that of kinase inhibitors. As such, commercial curation of these compounds is limited. Despite these limitations, the 24-member Phosphatase Inhibitor Library Set from TargetMol (Wellesley Hills, MA), along with the Enzo Screen-Well Phosphatase Inhibitor Library (Farmingdale, NY) provided a total of 48 unique structures. Each was dosed in monotherapy to a final concentration of 20 μ M, and then co-administered at that same concentration along with colistin at 1/16th of each strain's respective colistin MIC. Any compound that rendered bacteria susceptible to the combination without antimicrobial activity in monotherapy was recorded as a hit molecule. This method was applied to a total of eight Gram-negative strains, four colistin-resistant (*A. baumannii* 4106, *K. pneumoniae* B9, *A. baumannii* ATCC 17978^{mcr-1}, and *Escherichia coli* ATCC 25922^{mcr-1}) and four colistin sensitive (*A. baumannii* ATCC 17978, *E. coli* ATCC 25922, *A. baumannii* 5075, and *P. aeruginosa* PAO1). In all, nine compounds had activity in at least one of these strains. All hit molecules were then subjected to a colistin MIC shift assay by dosing each hit molecule at 5 μ M to identify the most active molecules (Supplementary Information, Tables S1 and S2).

2.2 Identification of Most Active Structures

From nine active molecules, the field was narrowed to seven compounds that had activity in at least two strains (**1** – **7**, Figure 1). Each compound was then tested for stand alone antimicrobial activity and the ability to affect colistin sensitivity in fifteen additional strains to further determine the compounds with ubiquitous activity. We began by evaluating activity in seven highly colistin-resistant primary clinical isolates, four strains of *A. baumannii* (AB3941, AB3942, AB4106, and AB4112), and three of *K. pneumoniae* (KPA5, KPB9, KPC3). All seven of these strains return colistin MICs of at least 256 μ g/mL, at times exceeding 1024 μ g/mL, which is well above the CLSI colistin breakpoint MIC of 2 μ g/mL. As given in Table 1, all seven members of the compound panel returned measurable reductions in colistin MIC in at least five of the seven strains when dosed at 20 μ M. Of note, **1** – **7** were also non-antimicrobial in monotherapy at concentrations above 200 μ M with few exceptions (Table 1). Compounds **4** and **6** emerged as the most effective agents against *A. baumannii*, each returning upwards of a 2048-fold reduction in colistin MIC and definitively restoring polymyxin susceptibility. However, **4** became the compound of choice as **6** did not show activity against two of the three colistin-resistant strains of *K. pneumoniae*.

This process was repeated with two genetically engineered colistin-resistant strains (*A. baumannii* ATCC 17978^{mcr-1}, and *E. coli* ATCC 25922^{mcr-1}) (Y. Y. Liu et al., 2017) and their corresponding parent strains (*A. baumannii* ATCC 17978, and *E. coli* ATCC 25922). These engineered strains have each been transfected with a plasmid carrying the *mcr-1* gene that encodes a lipid A phosphoethanolamine transferase. In these strains, **4** once again restores colistin susceptibility, returning between an 8 to 32-fold reduction in colistin MIC when dosed at 20 μ M (Table 2). Intriguingly, **4** was inactive in the corresponding colistin-susceptible parent strains. Instead, **1** further potentiated colistin in both parent strains, returning 32 and 16-fold reductions in colistin MIC in AB17978 and EC25922, respectively.

To further investigate this trend, **1** – **7** were then tested in four additional colistin-susceptible Gram-negative isolates (*A. baumannii* 5075, *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* PAO1) (Table 3). When dosed at 20 μ M, **1** continued to display colistin potentiation and returned a 32-fold reduction in colistin MIC in both *A. baumannii* isolates, while showing least potency in PAO1, returning a four-fold reduction.

Evaluating **1** – **7** in each of these fifteen strains reinforced a trend that was first established in our pilot screening of eukaryotic kinase inhibitors. In the context of drug repurposing, reversing colistin resistance and potentiating colistin in polymyxin sensitive strains are typically (but not always) a mutually exclusive processes when the adjuvants are themselves non-toxic, and each require two completely different molecules. In our previous kinase inhibitor report, the celecoxib derivative AR-12 was the compound of choice for potentiating colistin against colistin-sensitive bacteria, while the salicylamide IMD 0354 broke colistin resistance. As with **1** and **4**, neither AR-12 nor IMD 0354 were able to simultaneously reverse colistin resistance and further potentiate the antibiotic.

2.3 Further Studies of Compounds **1** and **4**

To further evaluate the activity of both **1** and **4**, we first performed a series of dose-response studies, using two representative strains for colistin sensitive (AB5075/KP43816) and colistin resistant (AB4106 and KPB9) bacteria. As shown in Tables 4 and 5, ascomycin (**1**) returns dose-dependent activity in both colistin-sensitive isolates (Table 4), while **4** returns a dose-response in both highly colistin-resistant primary clinical isolates (Table 5). These same four strains were again used to explore the possibility that **1** or **4** could be acting as pan-assay interference compounds (PAINS). (J. B. Baell & Holloway, 2010; Jonathan B. Baell & Nissink, 2018) To rule out a non-specific aggregation effect, the activities of **1** and **4** were measured in media spiked with 0.001% Triton X100, and activity was retained in each case (Supplementary Information, Tables S3 and S4). We also performed the MIC shift assay in media supplemented with 10% Fetal Bovine Serum. In this case **1** retained activity while **4** was rendered inactive (Supplementary Information, Tables S5 and S6).

Next, we evaluated the effect of each compound on membrane permeability using Invitrogen's BacLight Live/Dead Assay kit that allows cell membrane permeability to be quantified by comparing the fluorescence ratio of readily permeable STYO-9 to that of propidium iodide, which can only enter bacterial cells with membrane damage. (Boulos, Prevost, Barbeau, Coallier, & Desjardins, 1999) Both **1** and **4** were subjected to this assay in

AB4106 and AB5075 to determine if either compound increased membrane permeability. In both strains, **1** returned no measurable change in membrane permeability, while **4** increased permeability minimally, returning a factor of 1.15 in AB4106 and 1.34 in AB5075.

Though colistin stands alone as a highly effective antibiotic, it is also known to increase cell membrane permeability at sub-MIC concentrations, and can act as an adjuvant for other molecules. It was therefore important to distinguish the principle agent of bacterial death in each adjuvant-antibiotic pairing. This can be accomplished by substituting colistin with polymyxin B nonapeptide (PMBN). Unlike colistin, PMBN lacks a fatty acyl tail appendage and does not have bactericidal activity but retains the ability to permeabilize bacterial cell membranes.(Ofek et al., 1994) In an inverse MIC shift assay where either colistin or PMBN is treated as the adjuvant, any potential reduction of the MIC of **1** or **4** seen in co-treatment with colistin and PMBN would suggest that polymyxins synergize with **1** or **4** by allowing them to enter the cell and act as antibiotics. As expected, a sub-MIC dose of colistin returns an apparent reduction in the MIC of both **1** and **4** in AB5075, but this does not occur with equimolar concentrations of PMBN (Supplementary Information, Tables S7 and S8). This suggests that both **1** and **4** are indeed adjuvants, while colistin retains its role as the antibiotic.

Previous disclosures have discussed synergy between colistin and macrolide antibiotics, with azithromycin having greatest activity.(Li et al., 2018) Ascomycin (**1**) is a macrolide but does not have antimicrobial effects. As such we sought to conduct an equimolar comparison of **1** to a panel of macrolide antibiotics including azithromycin, clarithromycin, erythromycin, as well as the glycopeptide vancomycin. As shown in Table 6, **1** achieves a 32-fold reduction in colistin MIC in AB5075, and a 16-fold reduction in KPB2 at 20 μ M, whereas the other antibiotics return at best eight-fold reductions in colistin MIC at the same concentration. In a separate assay, **1** and **4** were both evaluated for potential adjuvant effects with six other antibiotics (Supplementary Information, Table S9). Neither compound returned an MIC shift of any of these antibiotics in AB5075, suggesting that both have activity that is specific to polymyxins.

2.4 Adjuvant Effects on Bacterial Growth

The persistent ability of bacteria to adapt to harsh environments cannot be overstated and it should be assumed that any given bacterial species will eventually develop resistance to molecules with antimicrobial properties. Therefore, it is important to minimize antimicrobial effects when selecting antibiotic adjuvant candidates to potentially slow resistance acquisition. To quantify activity further, we constructed bacterial growth curves in the absence and presence of adjuvants +/- colistin in AB5075 and AB4106. As shown in Figures 2 and 3, both **1** and **4** do not display an antimicrobial effect when growth is compared to an untreated culture, and each displays dose dependent cell death when combined with colistin.

2.5 Eukaryotic Cell Toxicity

Quantification of eukaryotic cell toxicity is an important step in the drug repurposing process. Often, each molecule in a commercially available screening library has already

been tested in a number of cell-based assays. However, for the purposes of this study, the potential for increased cell toxicity when each adjuvant is combined with colistin was explored using the 4T1 mouse mammary tumor cell line (ATCC, Manassas, VA). Both **1** and **4** were dosed in monotherapy and in combination with a therapeutic dose of colistin (1 $\mu\text{g/mL}$) for 18 hours. Cell viability was then determined by exposing cells to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In healthy cells, mitochondria convert the tetrazolium core of MTT to its corresponding formazan product forming insoluble purple crystals that afford a colorimetric quantification of cell death. Both **1** and **4** returned CT_{50} values above their active concentrations (110.2 μM and 114.1 μM , respectively), and cotreatment with colistin did not increase the toxicity of either compound (204 μM and 101.3 μM , respectively) (Figure 4).

In summation, this report further reinforces that molecules with known eukaryotic activity can serve as a source for the discovery of antibiotic adjuvants. Ultimately, this study identified two natural products that have synergy with colistin. The non-antimicrobial macrolide ascomycin (**1**) consistently potentiated colistin in a number of Gram-negative strains while outperforming other macrolides. Kuwanon G (**4**) showed consistent potency in several highly colistin-resistant clinical isolates and restored colistin MICs below the CLSI breakpoint. Further assays showed that both **1** and **4** had dose dependent activity, had no effects on bacterial growth in monotherapy, and do not act through aggregation. In previous studies from our group, molecules that break colistin resistance significantly downregulate *pmrCAB* expression as measured by quantitative polymerase chain reaction (qPCR) assays. (Harris et al., 2014) Intriguingly, neither **1** nor **4** displayed this activity (Supplementary Information, Figures S1-S4). Further investigations into each molecule's respective mechanism of action are thus warranted. In all, this report offers two new scaffolds that serve as colistin adjuvants and future demonstrates that drug repurposing efforts can identify molecule pools with increased diversity.

3. Experimental Methods

Phosphatase Inhibitor Library Screening.

Two phosphatase inhibitor libraries were utilized in this study: the TargetMol Phosphatase Inhibitor Library Set (Wellesley Hills, MA) and the Enzo Screen-Well Phosphatase Inhibitor Library (Farmingdale, NY). Compounds from each library were diluted to 1 mM in DMSO for dosing. Bacterial strains of interest were cultured for four hours in Cation-adjusted Muller Hinton Broth (CAMHB), subcultured to 5×10^5 CFU/mL and divided in 100 μL aliquots on a 96 well plate. Kinase inhibitors were then spiked in media to a final concentration of 20 μM , and either co-dosed with colistin to a desired concentration or given as monotherapy. Each respective condition was performed in duplicate and all wells returning a consensus lack of turbidity in dual therapy with colistin without toxicity in monotherapy were recorded as hit molecules.

Single-Compound Bacterial Susceptibility Assay.

This procedure follows the guidelines set by the Clinical Laboratory Sciences Institute. (CLSI, 2018) Bacteria were cultured for 4 to 6 hours in CAMHB and subcultured to 5×10^5

CFU/mL in fresh CAMHB. For each compound to be tested, a 1 mL aliquot of subculture was collected and dosed with a compound of interest to a final concentration of 200 μ M. Samples were then dispensed (200 μ L) into the first row of a 96-well microtiter plate in which subsequent wells were prefilled with 100 μ L of subculture. 100 μ L of dosed subculture was then serially diluted a total of 6 times in each subsequent row of the plate save for the last row for a control. Plates were then sealed and incubated stationary at 37 °C. After 18 hours, the plates were removed and MIC values were recorded. All compounds tested had a purity of >95%.

MIC Shift Assay.

This procedure was adapted from the guidelines set by the Clinical Laboratory Sciences Institute.(CLSI, 2018) Bacteria were cultured for 4 to 6 hours in CAMHB and subcultured to 5×10^5 CFU/mL in fresh CAMHB. For each compound to be tested, a 5 mL aliquot of subculture was taken and dosed with adjuvant from a DMSO stock. A 1 mL aliquot of each dosed subculture was collected and dosed with antibiotic to a set concentration. Co-dosed aliquots were then dispensed (200 μ L) into the first row of a 96-well microtiter plate in which subsequent wells were prefilled with 100 μ L of the corresponding dosed subculture. 100 μ L of co-dosed subculture was then serially diluted a total of 6 times in each subsequent row of the plate, save the last row as a control to afford serial dilution of the antibiotic while holding a constant concentration of adjuvant. Plates were then sealed and incubated stationary at 37 °C. After 18 hours, the plates were removed and MIC values were recorded. All compounds tested had a purity of >95%.

BacLight Cell Permeability Assay:

Bacteria were cultured overnight in CAMHB at 37°C and diluted 1:10 in fresh CAMHB and grown for an additional 4 hours to an OD₆₀₀ of ~0.3. Cultures were centrifuged at 10,000 g for 15 mins, supernatants were discarded, and cell pellets were washed once with sterile water and resuspended in 1/10th the original volume in sterile water or water dosed with compounds. Suspensions were incubated for 1 hour at 37°C with shaking and centrifuged at 10,000 g for 15 mins, washed with sterile water and resuspended in sterile water supplemented with 1:1 SYTO-9 and propidium iodide (3 μ L/mL, from Invitrogen BacLight Kit). 100 μ L of each test condition were added to a 96 well plate which was covered and allowed to stand at room temperature. After 15 mins, the plate was read at 530 nm and 645 nm (excitation 485 nm). The ratio of green to red fluorescence was calculated as a percentage of the control.

Time-dependent Bacterial Growth Quantification.

Strains were cultured for 18 hours in CAMHB and subcultured to 5×10^5 CFU/mL in fresh CAMHB. The subculture was then transferred to culture tubes in 5 mL aliquots, which were dosed with adjuvant to a set concentration, or adjuvants co-administered with colistin, save one aliquot as a control. All subcultures were then incubated at 37 °C with shaking. At 2, 4, 6, 8, and 24 hours post subculture, 100 μ L samples of each condition were serially-diluted in 900 μ L aliquots of CAMHB for a total of 5 to 7 times. 100 μ L of each dilution point was then plated on LB agar and incubated overnight. The total number of bacterial colonies on each plate were recorded.

Quantitative PCR.

Bacteria were cultured overnight in CAMHB at 37 °C with shaking and diluted 1:20 in either in fresh CAMHB, or fresh CAMHB spiked with compounds of interest. After the desired incubation time, cultures were centrifuged at 5 °C and 3,000 rpm for 5 mins. Supernatants were discarded and cell pellets were resuspended in 1 mL of RNA Stat-60 lysis buffer (AMS Biotechnology, Abingdon-on-Thames, UK). RNA was isolated according to the manufacturer's protocol and resuspended in sterile water supplemented with 0.0001% diethylpyrocarbonate (DEPC). The final concentration of RNA in each sample was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Two micrograms of cDNA were made from each sample using the High-Capacity Reverse Transcriptase Kit from Applied Biosystems (Foster City, CA) using the manufacturer's supplied reagents and protocols. Quantitative PCR reactions were then made up in 15 µL aliquots using 2x Bio-Rad SYBR Green Master Mix (Hercules, CA). All qPCR primers were used as described by Harris.(Harris et al., 2014) Five µL of each reaction was plated in duplicate on a sterile 384-well plate and sealed with optically clear film. Quantitative PCR was performed on an Applied Biosystems QuantStudio 6 real-time PCR machine and analyzed using the manufacturer's software. Ct values were normalized to 16S RNA and converted to fold-change values by the ddCT method.

Eukaryotic Cell Toxicity.

4T1 cells (ATCC, Manassas, VA) were plated at a density of 1×10^4 cells/well in 96-well plates in Roswell Park Memorial Institute Media 1640 (RPMI) (Gibco, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (Gibco), 2 mM GlutaMAX (Gibco) and 50 µM 2-mercaptoethanol (Sigma Aldrich, St. Louis, MO) and incubated at 37°C under a 5% CO₂ atmosphere in the dark for 18 h. Cell cultures were treated with serial dilutions of compounds in the presence or absence of 1 µg/mL colistin (3 replicates per condition) and incubated for an additional 18 hours. The following control conditions were used: media only (blank), 1% Triton X100 (0% cell viability), 0.5% DMSO (100% cell viability). Each condition was then treated with 10% volume of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich) in sterile filtered 1X phosphate buffered saline (PBS) and incubated for 2 h. at 37° C in 5% CO₂, after which the media was aspirated and the resulting formazan crystals were resuspended in 100 µL acidified (4 mM HCl) isopropanol. The 96-well plate was then read at 540 nm on a FLUOstar Optima (BMG Labtech Cary, NC) microplate reader. Cell viability was calculated as a percentage using the two previously mentioned controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of adjuvants candidates: ascomycin (**1**); F1063-0967 (**2**); SF 1670 (**3**); kuwanon G (**4**); PRL-3 inhibitor 1 (**5**); 9,10-phenanthrenequinone (**6**); sanguinarine chloride (**7**).

Figure 2.
Time-dependent growth of AB 5075 treated with **1** alone and **1** in combination with various concentrations of colistin.

Figure 3.
Time-dependent growth of AB 4106 treated with **4** alone and **4** in combination with various concentrations of colistin.

Figure 4.
Cell toxicity curves of 4T1 cells treated with **1** and **4** alone or in combination with 1 $\mu\text{g/mL}$ of colistin.

Table 1.

Colistin MIC ($\mu\text{g/mL}$ (fold reduction)) of seven colistin-resistant strains of *A. baumannii* (AB), and *K. pneumoniae* (KP) co-dosed with 20 μM adjuvant and colistin. All adjuvant MICs are $>200 \mu\text{M}$ unless noted.

	Colistin MIC	1	2	3	4	5	6	7
AB3941	512 $\mu\text{g/mL}$	64 (8)	256 (2)	32 (16)	1 (512) **	32 (16)	0.5 (1024) *	16 (32) *
AB3942	512 $\mu\text{g/mL}$	128 (4)	256 (2)	32 (16)	1 (512) **	32 (16)	0.5 (1024) *	32 (16) *
AB4106	1024 $\mu\text{g/mL}$	128 (8)	256 (4)	32 (32)	0.5 (2048) **	32 (32)	0.5 (2048) *	64 (16) *
AB4112	$>1024 \mu\text{g/mL}$	128 (8)	256 (4)	64 (16)	2 (512) *	64 (16)	1 (1024) *	64 (16) *
KPA5	$>1024 \mu\text{g/mL}$	>1024 (0)	>1024 (0)	>1024 (0)	8 (>128)	512 (>2)	>1024 (0)	64 (>16) *
KPB9	1024 $\mu\text{g/mL}$	4 (256)	8 (128)	32 (32)	1 (1024)	32 (32)	8 (128)	16 (64) **
KPC3	256 $\mu\text{g/mL}$	256 (2)	64 (8)	128 (4)	16 (32)	16 (16)	256 (0)	256 (0) *

* Compound MIC = 200 μM ;

** Compound MIC = 100 μM

Table 2.

Colistin MIC ($\mu\text{g/mL}$ (fold reduction)) of two colistin-resistant *mcr-1* positive strains of *A. baumannii* (AB), and *E. coli* (EC) and their corresponding parent strains co-dosed with 20 μM adjuvant and colistin. All adjuvant MICs are $>200 \mu\text{M}$ unless noted.

	Colistin MIC	1	2	3	4	5	6	7
AB17978 ^{+mcr-1}	16 $\mu\text{g/mL}$	16 (0)	16 (0)	4 (4) [*]	0.5 (32)	4 (4)	0.25 (64)	4 (4) [*]
EC25922 ^{+mcr-1}	8 $\mu\text{g/mL}$	2 (4)	8 (0)	8 (0)	1 (8)	4 (2)	2 (4)	4 (2) ^{**}
AB17978	1 $\mu\text{g/mL}$	0.0625 (16)	1 (0)	1 (0) [*]	0.5 (2)	1 (0)	1 (0)	1 (0) [*]
EC25922	0.5 $\mu\text{g/mL}$	0.0156 (32)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0)	0.06125 (8) ^{**}

^{*} Compound MIC = 200 μM ;

^{**} Compound MIC = 100 μM

Table 3.

Colistin MIC ($\mu\text{g/mL}$ (fold reduction)) of four colistin-sensitive isolates of *A. baumannii* (AB), *K. pneumoniae* (KP), and *P. aeruginosa* (PA) co-dosed with 20 μM adjuvant and colistin. All adjuvant MICs are $>200 \mu\text{M}$ unless noted.

	Colistin MIC	1	2	3	4	5	6	7
AB5075	1 $\mu\text{g/mL}$	0.03125 (32)	1 (0)	0.5 (2)	0.5 (2) *	1 (0)	0.5 (2)	0.5 (2) **
AB19606	1 $\mu\text{g/mL}$	0.03125 (32)	1 (0)	0.5 (2)	1 (0) *	1 (0)	0.5 (2)	0.5 (2) **
KP43816	0.5 $\mu\text{g/mL}$	0.0625 (8)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0)	0.5 (0) **
PAO1	2 $\mu\text{g/mL}$	0.5 (4)	2 (0)	2 (0)	2 (0)	2 (0)	2 (0)	2 (0) **

* Compound MIC = 200 μM ;

** Compound MIC = 100 μM

Table 4.

Dose-dependent activity of **1** in two colistin sensitive strains given as colistin MIC ((ng/mL) (fold-reduction)).

	AB5075	KP43816
No Compound	1000 ng/mL	500 ng/mL
+ 20 μ M 1	31.3 (32)	62.5 (8)
+ 15 μ M 1	31.3 (32)	125 (4)
+ 10 μ M 1	62.5 (16)	125 (4)
+ 5 μ M 1	250 (4)	250 (2)

Table 5.

Dose-dependent activity of **1** in two colistin sensitive strains given as colistin MIC (($\mu\text{g/mL}$) (fold-reduction)).

	AB4106	KPB9
No Compound	1024 $\mu\text{g/mL}$	1024 $\mu\text{g/mL}$
+ 20 μM 1	0.5 (2048)	1 (1024)
+ 15 μM 1	1 (1024)	4 (256)
+ 10 μM 1	4 (256)	8 (128)
+ 5 μM 1	64 (16)	128 (8)

Table 6.

Colistin MIC (ng/mL (fold-reduction)) of polymyxin-sensitive strains tested with a panel of macrolides dosed at 20 μ M.

	AB5075	KPB2
No Compound	1000 ng/mL	500 ng/mL
Ascomycin	31.3 (32)	31.3 (16)
Azithromycin	125 (8)	250 (2)
Clarithromycin	125 (8)	250 (2)
Erythromycin	250 (4)	250 (2)
Vancomycin	500 (2)	500 (0)