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Abstract

Extrusion of drugs or drug-like compounds through bacterial efflux pumps is a serious health issue that leads to loss in drug efficacy. Combinatorial therapies of low-efficacy drugs with efflux pump inhibitors may help to restore the activities of such drugs. In this quest, natural products are attractive molecules, since in addition to their wide range of bioactivities they may inhibit efflux pumps. The current work repurposed the bioactive alkaloid roemerine as a potential efflux pump inhibitor. In *Bacillus subtilis*, both Bmr and BmrA, belonging to the major facilitator and the ATP-binding cassette superfamilies, respectively, were found to be inhibited by roemerine. Scanning electron microscopy and RNA-Seq analyses showed that it potentiated the effect of berberine. Growth rates and checkerboard assays confirmed the synergy of roemerine and berberine and that roemerine prevented berberine efflux by inhibiting Bmr. Transport assays with inverted membrane vesicles prepared from *Escherichia coli* overexpressing BmrA showed that increasing roemerine concentration decreased the transport of doxorubicin, the BmrA substrate, confirming that roemerine may also be considered as an inhibitor of BmrA. Thus, these findings suggest that conjugation of roemerine to substrates of efflux pumps, Bmr and BmrA, may help to potentiate the activity of their drug substrates.

Keywords: Efflux pump inhibition, alkaloid, roemerine, berberine, Bmr, BmrA.

1. Introduction

Bacterial recognition of foreign molecules, such as drugs or drug-like compounds, leads to their extrusion through multidrug efflux pumps [1]. Minimization of the intracellular concentration of an antibacterial agent through the action of these pumps constitutes a serious health issue as it notably reduces the efficacy of the drugs used. The decrease in the pace of 'new antibacterial molecule discovery' raises interest to restore the activities of existing molecules by inhibiting the actions of efflux pumps. Thus, combinatorial therapies of drugs with efflux pump inhibitors (EPIs) have the potential to bring molecules of low efficacy back to the clinic.

Many natural products bear more than one biological activity as a consequence of their multi-target properties [2]. To date, a significant number of natural compounds have been reported to possess EPI properties [3], in addition to their known anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor, anti-oxidant, anti-depressant, anti-cancer, anti-diarrheal, cholagogue, hepatoprotective, and anti-diabetic activities [4]. These natural compounds include, but are not limited to, alkaloids such as reserpine and piperine, essential oils, phenolic metabolites such as silibinin, 5'-methoxy-hydnocarpin (5'-MHC), and catechin gallates [5–7]. Many reports have proven that, due to their multi-target properties, most of these molecules usually not only inhibit one but more than one pump in a cell [8]. Thus, finding the actual target might not be a trivial task, even after the establishment of the EPI activity.

The alkaloid reserpine of the indole family is among the first natural compounds to be identified as an EPI. Originally, it was known for its antipsychotic and antihypertensive drug activities. Later studies have shown that it also targets efflux pumps, such as BmrA [9], Bmr, NorA [10], PatA/PatB [11], and TetK [12] significantly enhancing the activities of antibacterial agents [13,14]. Based on the clues that many alkaloids possess a multi-target feature, we hypothesized that other alkaloids may act as EPIs in bacterial cells. Information that the aporphine alkaloid roemerine enhanced the cytotoxic response mediated by vinblastine due to its possible

interaction with the eukaryotic efflux pump P-glycoprotein [15], has further motivated us to evaluate three structurally similar biologically active aporphine alkaloids (Fig. 1) as EPI candidates.

Name	1	2	3	6	9	10	11
Boldine	OCH_3	OH	H	CH_3	OH	OCH_3	H
Roemerine	–O–CH_2–O–		H	CH_3	H	H	H
Bulbocapnine	–O–CH_2–O–		H	CH_3	H	OCH_3	OH

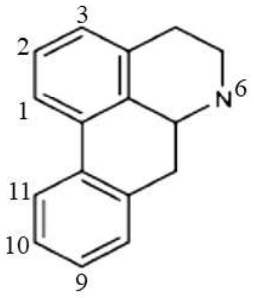

Fig. 1. Structures of alkaloids studied in this work.

These selected alkaloids display various biological activities by binding different targets, i.e. boldine has antioxidant, hepatoprotective, cytoprotective, antipyretic, and anti-inflammatory activities [16]; bulbocapnine inhibits acetylcholinesterase [17] and tyrosine hydroxylase activities [18] in addition to its involvement in the prevention of amyloid beta-protein fiber formation; roemerine acts as an inhibitor for CD45 protein tyrosine phosphatase [19] and exhibits antibacterial and antifungal activities [20]. Although these and many other alkaloids possess valuable bioactive properties, they may display cytotoxic properties when administered at their active concentrations. However, conjugation of these alkaloids as EPIs to other molecules may also help to reduce their effective concentrations.

The current study aimed to examine the EPI properties of selected alkaloids by observing the potentiation of the antibacterial activity of berberine when administered in combination with the selected EPI candidates. The antimicrobial berberine is a well-known substrate of efflux pumps and its activity is commonly impaired due to the activity of efflux pumps [21]. Thus, the synergistic activity of the alkaloid-berberine combinations will be sought in an attempt to find new EPIs.

2. Materials and methods

2.1. Bacterial strains

Wild-type *Bacillus subtilis* 168 (DSM 402), *B. subtilis ΔbmrA* [9] were used for testing the EPI properties of alkaloids. *Escherichia coli* C41(DE3) cells were used for overexpression of BmrA in transport assays [22].

2.2. Alkaloids used

Berberine chloride hydrate (CAS No. 141433-60-5), boldine (CAS No. 476-70-0), (+)-bulbocapnine hydrochloride (CAS No. 632-47-3) were obtained from Sigma-Aldrich. (-)-Roemerine was isolated from *Papaver syriacum* Boiss. et Blanche. All alkaloids were dissolved in dimethyl sulfoxide (DMSO).

2.3. Plant material and alkaloid extraction

Papaver syriacum Boiss. et Blanche was collected from Hatay (southern part of Turkey) in June 2016 (altitude 724 m). Voucher specimens were identified by Şükran Kültür and are deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE NO: 110346).

Aerial parts of *P. syriacum* (200 g) were dried and extracted with MeOH and the extract was concentrated under reduced pressure. The residue was taken up in 5% hydrochloric acid. The acid extract was first washed with light petroleum and then with diethyl ether. The aqueous layer was made alkaline with NH_4OH to pH 7-8 and extracted successively with $CHCl_3$. The combined $CHCl_3$ extracts were dried over anhydrous Na_2SO4, filtered and concentrated under vacuum to yield the tertiary alkaloid extracts. The tertiary alkaloid extract was separated on a column of silica gel (Kieselgel 60, 0.063-0.200 mm, 70-230 mesh) eluting with $CHCl_3$ and $CHCl_3$:MeOH (95:5, 90:10, 80:20). Fractions were evaporated and purified by preparative thin-layer chromatography on silica gel to afford pure alkaloids. The solvent system used for preparative chromatography was cyclohexane:chloroform:diethylamine (7:2:1). Alkaloid roemerine was obtained in the pure form and gave a positive reaction to Dragendorff reagent

which suggests the presence of an alkaloid. The identification of roemerine was carried out by comparing its UV, ^{1}H-NMR spectral data with values described in literature and TLC R_f values with an authentic sample [23]. UV spectrum was taken with Jasco V-530 spectrophotometer and ^{1}H-NMR spectra was recorded on a Varian Unity Inova 500 MHz spectrometer. The UV spectrum of roemerine showed absorptions at 233, 264 (sh), 271, 285 (sh), 315 nm, characteristic of aporphine alkaloids. The ^{1}H-NMR spectrum showed typical signals for aporphine alkaloids at: ($CDCl_3$, 500 MHz, δ) 2.45 ppm (3H, s, N-CH_3), 5.85-6.00 ppm (2H, dd, —OCH_2O methylenes), 6.48 ppm (1H, s, C-3), 7.06-7.25 ppm (3H, m, C-8-9-10), 7.98 ppm (1H, d, C-11). R_f values for roemerine were 0.79 in the solvent system cyclohexane:chloroform:diethylamine (7:2:1) and 0.72 in cyclohexane:diethylamine (8:2).

2.4. Bacterial growth conditions

B. subtilis cells were grown in nutrient broth (NB, Merck, Germany) at 37 °C and 180 rpm and treated with berberine 75 μg·mL^{-1} and/or 25 μg·mL^{-1} of different aporphine alkaloids. Drug treatment was achieved as OD_{600} of the culture broth reached 0.55±0.05. Alkaloids were dissolved in DMSO and control cells were treated with an equal volume of DMSO. Bacterial growth was monitored at OD_{600}.

2.5. Total RNA isolation

One-hour berberine (75 μg·mL^{-1}) and roemerine (25 μg·mL^{-1}) treated and control wild-type cells were placed in RNAprotect Bacteria Reagent (Qiagen, Germany) and incubated 5 minutes at room temperature. Cell suspensions were centrifuged at 5000x*g* for 10 minutes. Total RNA isolation was achieved using Qiagen RNeasy Mini Kit (Qiagen, Germany) with mechanical disruption at 6 m/s for 40 seconds (FastPrep-24 Instrument, MP Biomedicals, USA). The quality of the samples was checked using Agilent 2100 bioanalyzer [24].

2.6. Sequencing of mRNA

RNA-Seq of the samples was performed at the Beijing Genomics Institute (BGI, Shenzhen, China). The library was constructed with paired-end sequencing using Illumina HiSeqTM 4000 technology.

2.7. Analysis Pipeline

Analysis of the raw data has been carried out using R [25,26], as outlined previously [24]. Taking *B. subtilis* 168 genome (RefSeq accession number NC_000964.3) as the reference, differences with an absolute value of $\log_2 \geq 1$ were accepted as significant. Selected genes were classified according to the SubtiWiki database categories [27]. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE106296.

2.8. Validation using qPCR

Five differentially expressed genes were selected for validation with qPCR using 16S ribosomal RNA, *rrnA*-16S, as the housekeeping gene. cDNAs were synthesized using QuantiTech Reverse Transcription Kit (Qiagen, Germany). Reactions were carried out using the formerly designed primers, as explained previously [24]. Fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method [28]. All reactions were performed with at least two technical replicates.

2.9. Cell analysis with scanning electron microscopy

Samples (control and berberine-roemerine treated cells) for SEM were prepared based on our previously reported protocol [29]. SEM images were obtained by Philips XL30 ESEM-FEG/EDAX system (Philips, Holland) under high vacuum mode with 10 kV energy and 3.0 spot size.

2.10. Measurement of minimum inhibitory concentration and synergy

Minimum inhibitory concentration (MIC) was measured using broth dilution method [30]. Serial two-fold dilutions of the berberine and roemerine were prepared in sterile 96-well U-

bottomed plates; 50 µL of the cell culture (10^5 CFU/mL) was deposited in each well in a total volume of 100 µL. MIC was evaluated with reference to control cells, mixed with DMSO or fresh growth media.

Checkerboard assay has been performed to determine the synergy between berberine and roemerine. In a U-bottom 96-well plate, as berberine was two-fold serially diluted horizontally, roemerine was two-fold serially diluted vertically. Dilution was achieved with fresh NB. Concentrations were kept in the range of 8-512 µg·mL^{-1} for berberine and 4-256 µg·mL^{-1} for roemerine. A control lane was prepared with DMSO. Then each well was mixed with an equal volume of cell suspensions to get an initial concentration of 5×10^5 CFU/mL in each well. Plates were incubated at 37 °C for 24 hours without shaking.

The synergy between the compounds was evaluated by calculating the fractional inhibitory concentration index (FICI):

$$\text{FICI} = \sum_{i=1}^{n} \frac{MIC(antimicrobial\ agent\ in\ combination)}{MIC(antimicrobial\ agent\ alone)}$$

FICI of ≤ 0.5 means that the agents act synergistically; there is partial synergy when FICI is between 0.5 and 1.0; when FICI is =1.0, the effect is additive [31]. The MIC values were defined as the lowest concentrations showing no visible cell growth after 24 hours of incubation.

2.11. Transport assay with BmrA

Cloning, overexpression of recombinant BmrA in *E. coli* C41(DE3) cells, and preparation of inverted membrane vesicles were carried out as described previously [32]. The effect of roemerine on BmrA transport activity was monitored with Photon Technology International Quanta Master I Fluorimeter using doxorubicin as the substrate. The excitation and emission wavelengths were set at 480 and 590 nm, respectively, for doxorubicin.

Inverted membrane vesicles (100 µg of total protein) were added to a cuvette containing 50 mM Hepes-KOH (pH 8.0), 8.5 mM NaCl, 4 mM phosphoenolpyruvate, 60 µg of pyruvate kinase, 2

mM $MgCl_2$, and different concentrations of roemerine with a final volume of 1 mL. After incubation for 1 min at 25 ^oC, 10 μM of doxorubicin was added and its fluorescence was recorded for ~1 min. Then 2 mM ATP was added and the fluorescence intensity was monitored for several minutes.

3. Results and discussion

Berberine has long been known as a natural product with significant antibacterial activity against Gram-positive and Gram-negative species [29]. Unfortunately, intracellular berberine concentrations usually remain below the effective values due to the activity of drug efflux pumps [33].

The current work undertakes the effort to find new EPIs by increasing the antibacterial activity of berberine in the model Gram-positive microorganism *Bacillus subtilis*. With this aim, the potential of three aporphine type alkaloids of plant origin, roemerine, boldine, and bulbocapnine, was tested for their pump inhibitory activities and then for their possible pump targets.

3.1. Screening of the alkaloids for EPI properties

The initial screening for the efflux pump inhibitory potential of the alkaloids was achieved by monitoring growth after treatment of the cells with the selected compounds.

The working concentrations of the EPI candidates were set at 25 $\mu g \cdot mL^{-1}$, based on our previous report [24]. Roemerine itself acts as an antibacterial agent at high concentrations (>50 $\mu g \cdot mL^{-1}$) but at lower concentrations (<25 $\mu g \cdot mL^{-1}$), its presence did not affect cell growth [20,24]. Similarly, when *B. subtilis* cells were grown in the presence of 25 $\mu g \cdot mL^{-1}$ boldine or bulbocapnine, cell growth was not altered significantly (Fig. 2). While the growth rate of control cells was 0.65 hr^{-1}, the growth rates of the cells were constant between 0.59-0.63 hr^{-1} after roemerine, boldine, and bulbocapnine treatments.

Berberine working concentration was determined based on available literature. 250 μg·mL^{-1} berberine totally ceased growth while 100 μg·mL^{-1} significantly retarded growth [24]. On the other hand, 75 μg·mL^{-1} berberine decreased the growth rate to 0.53 hr^{-1} (Fig. 2). Our aim was to enhance the influence of berberine by conjugating it with an EPI, therefore 75 μg·mL^{-1} was selected for further work.

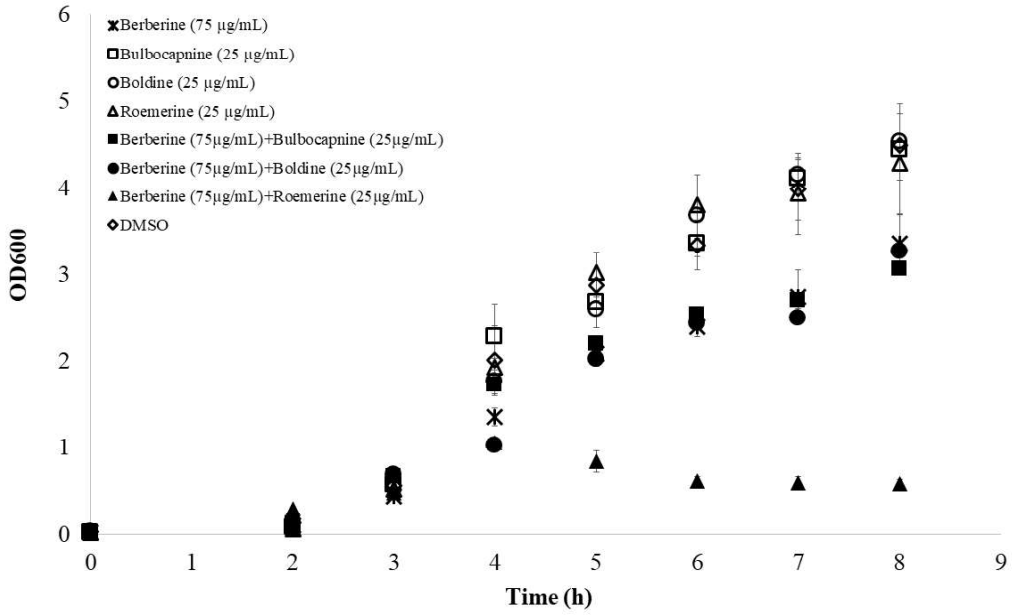

Fig. 2. Growth of wild-type *B. subtilis* cells in the presence of berberine and EPI candidates.

Finally, cells were treated with combinations of berberine and one of the alkaloids. Neither the berberine-boldine combination nor the berberine-bulbocapnine combination imposed a meaningful change on cell growth, in addition to that caused by only berberine. Growth rates lied between 0.42-0.45 hr^{-1} in the presence of berberine-boldine and berberine-bulbocapnine combinations. On the other hand, the berberine-roemerine combination exhibited a significant synergistic effect on the cells. Cell growth almost completely ceased (Fig. 2), a finding similar to that found with 250 μg·mL^{-1} berberine [24].

Berberine has long been known to be a substrate of drug efflux pumps. To enhance its biological activity, different strategies have been undertaken that includes the use of natural pump

inhibitors such as the flavolignan 5'-MHC [34] or conjugation of berberine to a multidrug efflux pump inhibitor [21,35]. Thus, the synergism observed for the berberine and roemerine combination is a strong indication for the involvement of roemerine in the inhibition of efflux pump activities in *B. subtilis*. However, the multi-target feature of alkaloids suggests that roemerine may target more than one efflux pump. Further work was carried out with the berberine-roemerine combination to verify roemerine's potential as an EPI and elucidate its targets.

3.2. Scanning electron microscopy analysis of the cells treated with the berberine-roemerine combination

The synergistic effect of berberine and roemerine on the cells was visualized by recording scanning electron microscopy (SEM) images (Fig. 3).

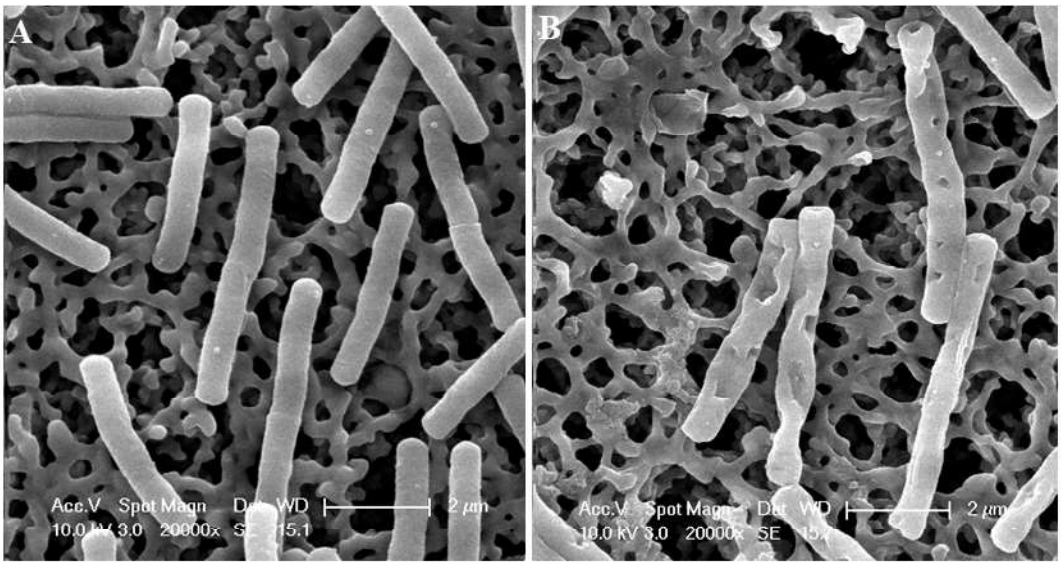

Fig. 3. Scanning electron microscopy images after one-hour treatment: A) control cells and B) cells with berberine (75 µg·mL^{-1})-roemerine (25 µg·mL^{-1}) combination treatment.

Control cells had shapes and sizes (5.2 ± 0.9 µm) characteristic of *Bacillus* species with smooth cell surfaces. After treatment of the cells with the berberine-roemerine combination, there were irregular dimples all over the surfaces. Furthermore, the cells were totally crumpled. The sizes

of a total of 21 selected cells seemed to cluster into two groups: in the first group were 10 cells which were significantly shorter (3.7 ± 0.5 μm) and in the second group were 11 cells which were longer (6.1 ± 0.5 μm) than the controls.

In our previous work, we have reported increased cell sizes (6.4 ± 1.2 μm) with dimples on cell surfaces after berberine treatment [24]. Increased size is a well-known consequence for the presence of berberine since it blocks cell division by inhibiting cell division protein, FtsZ [36]. However, the second group of cells was remarkably shorter than the control cells. It is possible that this could be the effect of roemerine. Indeed, when *B. subtilis* cells were treated with 75 $\mu g \cdot mL^{-1}$ of only roemerine, there was significant retardation in growth and the cells were found to be shorter with blebs on surfaces [24]. There were no blebs on the surfaces of cells treated with the berberine and roemerine combination. Thus, the antimicrobial effect of roemerine was only partial at that concentration.

When the cells were treated with the berberine-roemerine combination, we hypothesize that roemerine enhanced the effect of berberine by maintaining a higher intracellular berberine concentration. SEM images showed that extensively distributed dimples were bigger and deeper with the combination treatment.

3.3. Checkerboard assay of the berberine-roemerine combination

The synergistic effect of the berberine-roemerine combination was further validated by the checkerboard assay. The MIC values of berberine and roemerine for *B. subtilis* cells were 256 and 64 $\mu g \cdot mL^{-1}$, respectively (Table 1). The MIC values obtained for the Δ*bmrA* strain were identical with those obtained for the wild-type. On the other hand, for the Δ*bmr* strain, while the MIC value of roemerine remained unchanged, the MIC value of berberine dropped to 64 $\mu g \cdot mL^{-1}$. This sensitivity of the Δ*bmr* strain to berberine but not to roemerine strongly suggested that Bmr indeed effluxed berberine.

The checkerboard assay showed that, when administered together, the effective concentrations of berberine and roemerine dropped to 64 and 16 µg·mL^{-1}. With these concentrations, the FICI value was calculated as 0.5, meaning that these two alkaloids act synergistically.

Table 1. Summary of MIC of berberine and roemerine for different *B. subtilis* strains.

	MIC_{ber} (µg·mL^{-1})	**MIC_{roe} (µg·mL^{-1})**
B. subtilis 168	256	64
B. subtilis (Δ*bmr*)	64	64
B. subtilis (Δ*bmrA*)	256	64

3.4. Transcriptomic analysis of the synergy in B. subtilis 168

The synergistic effect of berberine and roemerine was then tackled by transcriptomic analysis. RNA-Seq results for the berberine-roemerine treated cells were comparatively analyzed with the available RNA-Seq data for control and berberine treated cells (GSE106296). Since the major task of using roemerine was to inhibit the efflux pumps, we speculated that the antimicrobial effect of berberine would be amplified in the cells treated with the combination. The statistics of the reads obtained from RNA-Seq analysis for the berberine and roemerine treated cells is given in Table 2. The transcripts were aligned taking *B. subtilis* 168 genome as the reference. Analysis has returned mapped reads over 90%.

Table 2. Summary of read statistics results for control and alkaloid treated *B. subtilis* cDNA samples.

Treatment	**Read length (bp)**	**Clean Reads**	**Clean bases**	**GC (%)**	**Q20 (%)**
DMSO*	100	11128658	1112865800	44.60	98.6
Berberine*	100	11083418	1108341800	43.91	93.2
Berberine and roemerine	100	11122254	1112225400	43.88	97.9

* Data taken from Avci et al. [24]

Taking the absolute value of $\log_2 \geq 1$ as significant, the reads were compared to the RNA-Seq obtained from control and 75 µg·mL^{-1} berberine treated *B. subtilis* cells (GSE104946). The

presence of the additional 25 μg·mL^{-1} roemerine almost doubled the number of affected genes. While a total of 1305 genes were differentially expressed in berberine treated cells, a total of 2317 genes were differentially expressed in the presence of the combination. Of these genes, 995 were common in both cells, which demonstrated that about 75% of the differentially expressed genes with berberine, were also differentially expressed with the combination treatment.

Differentially expressed genes were categorized based on the information in SubtiWiki database is presented in Fig. 4. Based on their functions, some of the genes appear in more than one category.

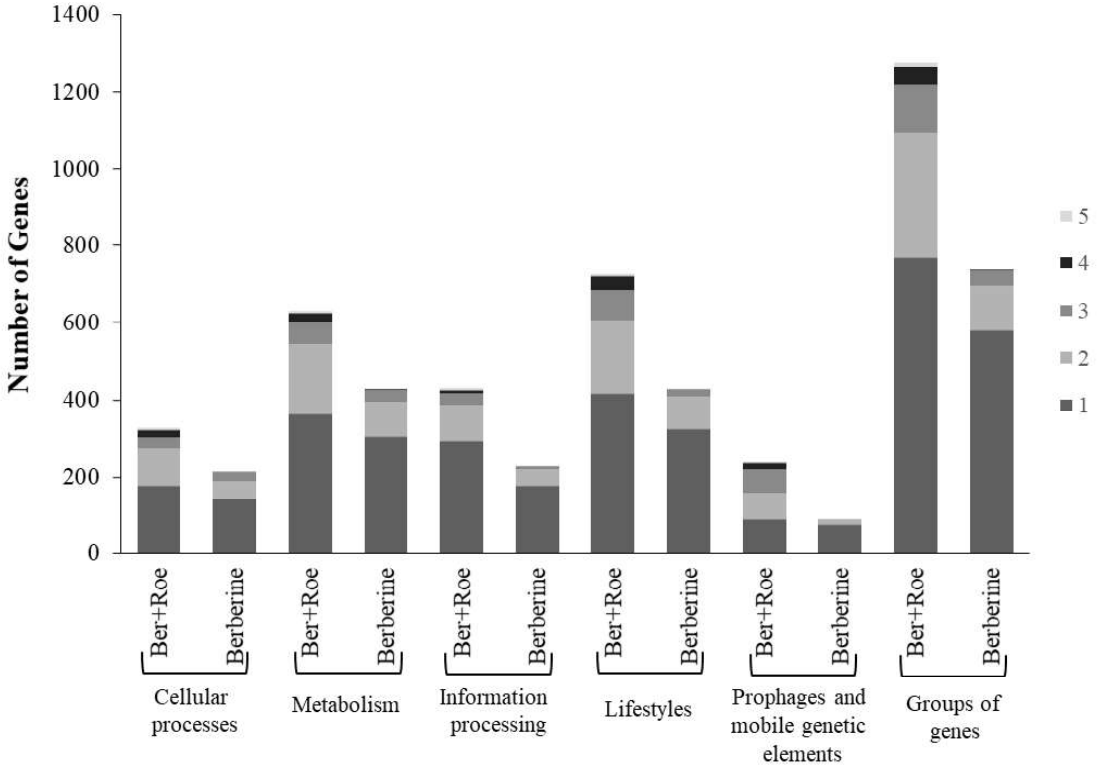

Fig. 4. Differentially expressed genes categorized based on the groupings in SubtiWiki database. The numbers that appear to the right of the plot indicate the absolute values of the fold changes in $\log_2$ scale.

In each category, the total number of affected genes was higher. Within each category, there

was a noticeable increase in the number of genes with a fold change of 4 or higher in $\log_2$ scale. This difference was highest in the category of prophages and mobile genetic elements but most of those were 'y-genes'; thus their functions were unknown.

The increase in the number of up-regulated genes involved in genetic competence (information processing category of Figure 4) as we switch from the berberine treatment to the combination treatment provided a clear overview of the turmoil within the cell. Genetic competence, the uptake of genetic material from the surroundings, might serve to allow the use of extracellular DNA for nutritional purposes but it simultaneously has a role in genetic recombination. The latter contributes to making cells tolerant to antibiotics [37]. While the struggle for survival was at a basal level with berberine, mainly due to its efflux, this struggle became increasingly important in the combination treatment. Roemerine blocked berberine efflux, enabling intracellular berberine accumulation. Consequently, by inducing the genes of genetic competence, cells seek a way to overcome this unfavorable condition.

We have previously reported that berberine changed the expression of genes associated with the membrane, its synthesis and structural re-organization. Furthermore, genes encoding membrane proteins of diverse functions were also among the affected ones [24]. Treatment of the cells with the combination not only increased the number of genes but also the absolute value of their $\log_2$ values (Fig. 4). Specifically, genes encoding proteins involved in cell wall synthesis, modification, turn-over, and recycling, genes encoding proteins of the biosynthesis of cell wall polymers, teichoic and teichuronic acids, and genes encoding proteins of the cell envelope such as carboxypeptidases, transpeptidases, and endopeptidases were among the highly affected targets.

An extensive number of genes encoding transporters, most of which were up-regulated, were affected with berberine treatment [24]. This number increased remarkably in the combination treatment. These transporters included but were not limited to, amino acid and oligopeptide

transporters, metal ion transporters, compatible solute transporters, and sugar transporters. Not only transporters of nutrient uptake but also transporters of TCA cycle intermediates (citrate, fumarate, malate, and alpha-keto-glutarate) seemed to be influenced especially under berberine-roemerine treatment as if the cells were in a state of nutrient limitation [38]. Since stress conditions were found to trigger the synthesis of various transporters [39], this finding was not unexpected.

A key strategy followed specifically by low G+C Gram-positive (i.e. *Firmicutes*) bacteria under harsh environmental conditions is spore formation, in addition to the increase in stress related proteins. The genes such as those encoding spore crust assembly, germination, and maturation of this tightly regulated network increased both in number and fold changes with the combination treatment. It may, however, take several hours for the cells to form such endospores [40] and, for this reason, most of the cells died before this process could take place, as evidenced by SEM images. Furthermore, berberine accumulation in the combination treatment also triggered the expression of many genes related to survival and protection against stress conditions.

RNA-Seq data were finally validated using quantitative polymerase chain reaction (qPCR). The five genes selected from different pathways showed that the regulation pattern of all the selected genes was consistent, though with variations in the absolute values of the fold changes (Table 3). These kinds of variations commonly arise as a result of statistics in RNA-Seq (i.e. the high variability across samples causes loss in statistical significance). Furthermore, in qPCR, genes are normalized with respect to the housekeeping gene whereas RNA-Seq uses internal normalization using geometrical averages.

Table 3. Expression levels of selected genes.

Gene name	Pathway involved	Regulation pattern /log2 fold change (qPCR / RNA-Seq)
dhbB	Fe uptake and metabolism	3.1 / 1.5
feuA	Fe uptake and metabolism	3.6 / 3.5
bmr	Multidrug transporter	6.2 / 1.1
narH	Anaerobic respiration and proton motive force (PMF) generation	BDL / -4.5
aroA	Aromatic amino acid biosynthesis	-0.9 / -2.5

BDL: Below detection limits

3.5.Identification of roemerine efflux pump targets

B. subtilis has an abundant number of well-characterized efflux pumps including BceAB [41], BmrA [9], BmrCD [42,43] of the ABC superfamily; Blt [44,45], Bmr [44–46], Bmr3 [47,48], LmrB [49,50], and MdtP [51] of the MFS superfamily; YerP [52] of resistance-nodulation-cell division (RND) superfamily, and EbrAB [53] of the small multidrug resistance (SMR) superfamily. Among these, Bmr is one of the first-discovered bacterial efflux pumps and Blt is another that is highly homologous to Bmr (52% sequence identity) [13,54].

The work by You et al. suggested that roemerine increases the cytotoxicity of vinblastine in multidrug-resistant KB-V1 cells by interacting with the eukaryotic multidrug resistance (MDR) pump, P-glycoprotein [15]. Since BmrA is the prokaryotic homologue of P-glycoprotein in *B. subtilis* [9], we speculated that roemerine could bind and inhibit this efflux pump. On the other hand, berberine has been proven to be a substrate of the NorA pump of *Staphylococcus aureus* [34]. Thus, Bmr of *B. subtilis*, a close homologue of NorA, might promote active berberine efflux in these cells [55,56]. Indeed both the MIC values and the synergism of berberine and roemerine point to the inhibition of Bmr by roemerine. Furthermore, since many pump inhibitors target more than one pump, both BmrA and Bmr may also be simultaneously inhibited by roemerine. To this end, we focused on the two KO mutants of *B. subtilis* cells,

Δ*bmr* and Δ*bmrA*, with the motivation to find whether these pumps are targeted by roemerine. The effect of the berberine-roemerine combination on the growth of these two mutants has been presented in Fig. 5.

The growth rates of the control and 25 μg·mL^{-1} roemerine treated mutant strains were similar to that of the wild-type, between 0.59-0.63 hr^{-1}. When the Δ*bmr*A strain was treated with 75 μg·mL^{-1} berberine alone, growth was only slightly retarded. Growth rate indeed decreased to 0.44 hr^{-1}, a slightly lower value than obtained with the wild-type cells. When this mutant strain was treated with the combination, growth almost completely ceased, as in the wild-type. The behavior of the Δ*bmr* strain was totally different; 75 μg·mL^{-1} berberine alone was sufficient to cease growth (Fig. 5). Actually, this could be foreseen since berberine concentration was already above the MIC.

Checkerboard assay and combination treatments (Fig. 2) together with growths of mutant strains (Fig. 5) strongly suggested that, in the wild-type strain, roemerine inhibited berberine efflux by blocking the Bmr pump. The absence of the Bmr pump facilitated the accumulation of higher intracellular berberine; thus, in the Δ*bmr* strain, berberine was able to display its antimicrobial effect at lower concentrations. Indeed, the MIC value of berberine obtained for the Δ*bmr* strain was 64 μg·mL^{-1} (Table 1), ¼MIC of the value obtained for the wild type.

The findings obtained with the Δ*bmrA* strain from the growth profiles were less obvious. The MIC values obtained for the Δ*bmrA* strain were identical with of the wild-type (Table 1). The effect of 75 μg·mL^{-1} berberine was somewhat superior in the Δ*bmrA* strain when compared to the wild type, which could mean that although the major berberine efflux is mediated through Bmr, a minor amount may possibly be effluxed through BmrA. An evidence for this was found also in the RNA-Seq analysis of the wild-type strain. Upon berberine and berberine-roemerine treatments, there was an expressional change of 1.5 and 1.1 ($\log_2$ scale) in Bmr, respectively, whereas the expressional change in BmrA was insignificant under the same

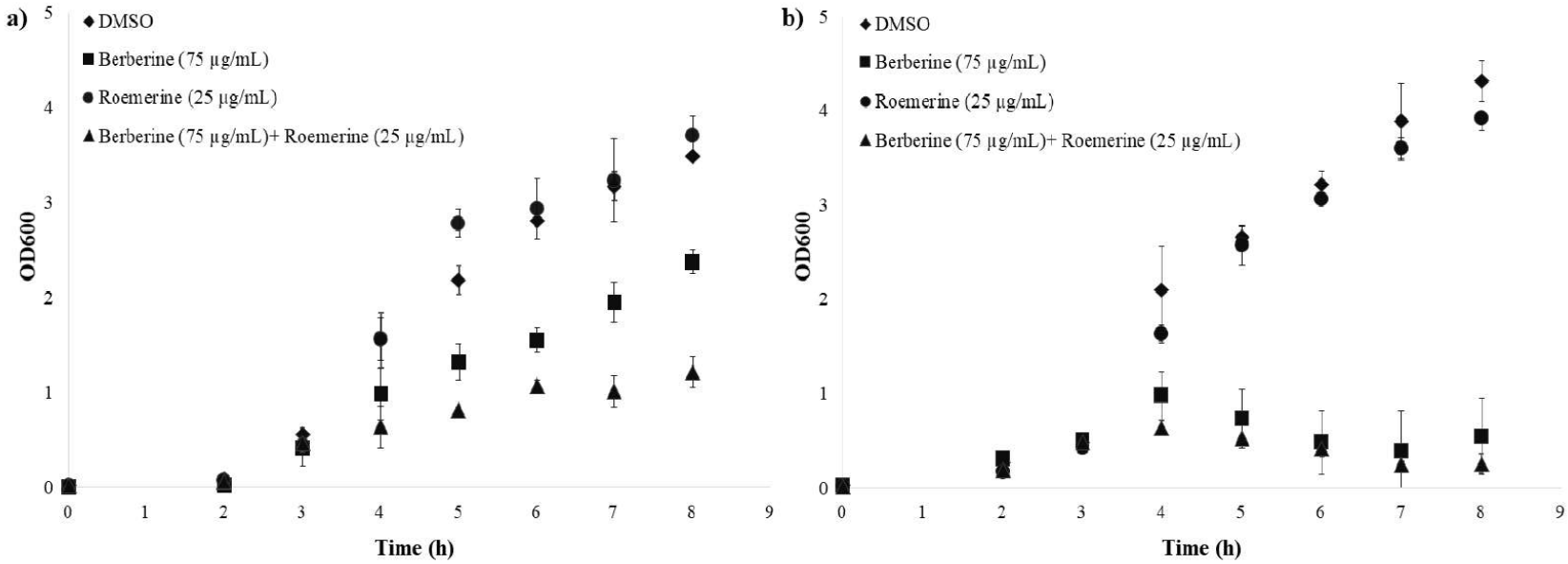

Fig. 5. Growth of Δ*bmrA* (a) and Δ*bmr* (b) strains under berberine-roemerine treatment.

conditions. Whereas BmrA was found to be constitutively expressed during the growth phase of *B. subtilis* [9], Bmr was found to be down-regulated by BmrR [57]. If the repression of Bmr by BmrR could be alleviated by berberine, this might explain the results found here.

The work with the mutant strains demonstrated that roemerine inhibited Bmr, but it was not sufficient to state whether or not roemerine inhibited the BmrA pump. Owing to the fact that the work with the mutants could not convey much information the inhibition of BmrA by roemerine, transport assays using BmrA overexpressed in *E. coli* inverted membrane vesicles were conducted. To this end, transport of the BmrA substrate doxorubicin was followed by measuring its fluorescence intensity. The findings showed that after incubation of the prepared membranes with increasing concentrations of roemerine, doxorubicin transport notably decreased (Fig. 6). This was a strong indication of the direct inhibition of BmrA by roemerine.

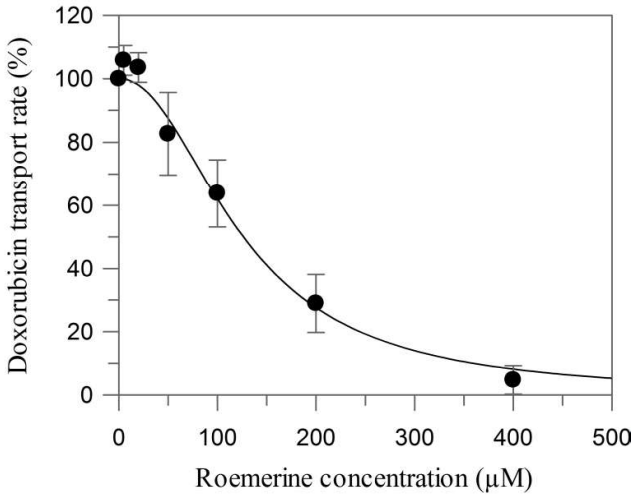

Fig. 6. Inhibition of doxorubicin transport by roemerine. *E. coli* inverted membrane vesicles overexpressing BmrA were assayed for doxorubicin (10 μM) transport in the presence of increasing concentrations of roemerine. Doxorubicin transport was measured by fluorescence, as previously described [9], and transport rates were calculated by initial slopes following ATP addition [22]. IC50 was calculated at 126 ± 8 μM with GraFit version 7 software. Data represent the average of 3 independent experiments with standard deviation.

4. Conclusions

MDR pumps are important contributors in all organisms for gaining resistance through extrusion of multiple compounds such as antibiotics and anticancer drugs [54,58]. This efflux minimizes the intracellular accumulation of drugs, increasing their effective concentrations. Thus, inhibition of MDR pumps is an attractive approach for revitalization of drugs of low efficacy due to their efflux.

The current work repurposed the bioactive alkaloid roemerine as a potential efflux pump inhibitor. In *B. subtilis* cells, both Bmr belonging to the MFS superfamily and BmrA belonging to the ABC superfamily were found to be inhibited by the bioactive alkaloid roemerine. SEM and RNA-Seq analysis showed that roemerine potentiated the effect of berberine by helping to maintain an effective intracellular berberine concentration. Evaluated growth rates and checkerboard assays confirmed that roemerine and berberine acted synergistically and that roemerine prevented berberine efflux by inhibiting the Bmr efflux pump. Finally, transport assays conducted using *E. coli* inverted membrane vesicles overexpressing BmrA confirmed that increasing concentrations of roemerine inhibited the transport of the BmrA substrate, doxorubicin, through this pump. Thus, we demonstrated that conjugation of roemerine to substrates of efflux pumps, Bmr and BmrA may potentiate the activity of these molecules. Furthermore, this would allow the administration of roemerine well below its cytotoxicity limits.

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Conflict of interest

The authors declare no conflict of interest**.**

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Conflict of interest

The authors declare no conflict of interest**.**