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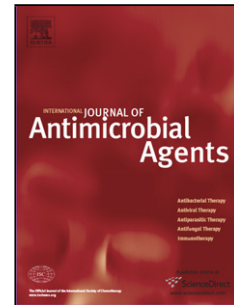
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Resistance of the *Burkholderia cepacia* complex to fosmidomycin and fosmidomycin derivatives

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ABSTRACT

The *Burkholderia cepacia* complex (BCC) is a group of 17 closely related opportunistic pathogens that are able to infect the respiratory tract of cystic fibrosis patients. BCC bacteria are intrinsically resistant to many antibiotics and are therefore difficult to eradicate. Fosmidomycin could be a new therapeutic agent to treat BCC infections as it inhibits 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr), a key enzyme in the non-mevalonate pathway essential in BCC bacteria for isoprenoid synthesis. In this study, the antimicrobial activity of fosmidomycin and eight fosmidomycin derivatives towards 40 BCC strains was investigated. All BCC strains were resistant to fosmidomycin, although addition of glucose-6-phosphate reduced the minimum inhibitory concentrations values of FR900098, the fosmidomycin acetyl derivative, from 512 mg/L to 64 mg/L for *Burkholderia multivorans* and *B. cepacia*. This enhanced activity was linked to increased expression of the genes involved in glycerol-3-phosphate transport, which appears to be the only route for fosmidomycin import in BCC bacteria. Furthermore, upregulation of a fosmidomycin resistance gene (*fsr*) encoding an efflux pump was observed during fosmidomycin and FR900098 treatment. These results strongly suggest that the observed resistance in BCC bacteria is due to insufficient uptake accompanied by fosmidomycin and FR900098 efflux.

1. Introduction

The *Burkholderia cepacia* complex (BCC) is a group of 17 closely related species. In the early 1980s, BCC bacteria emerged as problematic pathogens in cystic fibrosis (CF) patients, with *Burkholderia cenocepacia* and *Burkholderia multivorans* being the most frequently isolated species. Compared with *Pseudomonas aeruginosa*, BCC infections account for only a small percentage of respiratory infections within the CF population, however they are often associated with rapid deterioration of lung function and increased mortality [1]. Once infection with BCC bacteria is established, therapeutic options are limited because of the innate resistance of these bacteria to many antibiotics. Different resistance mechanisms have already been described, including increased efflux and decreased permeability of the outer membrane [2]. Despite these high levels of resistance, there has been little progress in developing new therapeutic strategies to fight these infections.

The non-mevalonate pathway for isoprenoid synthesis was discovered in the early 1990s as an alternative to the classical mevalonate pathway. Whilst most bacteria exclusively synthesise isoprenoids, such as coenzyme Q, through this pathway, mammalian cells do not possess it [3]. Fosmidomycin, a natural phosphonic acid antibiotic, is a specific inhibitor of a key enzyme of the non-mevalonate pathway, i.e. 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr). Although it has shown good safety and efficacy, e.g. for the treatment of uncomplicated *Plasmodium falciparum* malaria [4], rapid development of resistance during therapy is of great concern. White et al. [5] suggest that this resistance is due to decreased uptake of the compound. Uptake of fosmidomycin is hypothesised to follow the same route as its structural analogue fosfomycin, either through hexose phosphate or through

glycerol-3-phosphate transporters, as mutations in genes encoding these transport systems confer resistance to both fosmidomycin and fosfomicin [6].

Furthermore, the *B. cenocepacia* genome contains a fosmidomycin resistance gene (*fsr*) encoding an efflux pump that transports fosmidomycin out of the cell [7].

The aim of the present study was to evaluate the in vitro activity of fosmidomycin and eight derivatives against BCC and to elucidate the mechanisms of resistance in this group of organisms.

2. Materials and methods

2.1. Strains

The following 40 BCC strains were used: *B. cepacia* LMG 18821 and LMG 1222^T; *B. multivorans* LMG 18822, LMG 18825, LMG 13010^T and LMG 17588; *B. cenocepacia* LMG 16656^T, LMG 18828, LMG 18829, LMG 18830, LMG 6986 and K56-2; *Burkholderia stabilis* LMG 14294^T and LMG 14086; *Burkholderia vietnamiensis* LMG 18835 and LMG 10929^T; *Burkholderia dolosa* LMG 18943^T and LMG 18941; *Burkholderia ambifaria* LMG 19182^T and LMG 19467; *Burkholderia anthina* LMG 20980^T and LMG 20983; *Burkholderia pyrrocinia* LMG 14191^T and LMG 21824; *Burkholderia ubonensis* LMG 20358^T and LMG 24263; *Burkholderia latens* R-11768 and LMG 24264; *Burkholderia diffusa* LMG 24065^T and LMG 24266; *Burkholderia arboris* LMG 24066^T and R-132; *Burkholderia seminalis* LMG 24067^T and LMG 24272; *Burkholderia metallica* LMG 24068^T and R-2712; *Burkholderia lata* LMG 6992 and R-9940; and *Burkholderia contaminans* LMG 16227 and R-12710. All strains

were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were kindly provided by Dr P. Vandamme (Ghent University, Belgium). Two *Escherichia coli* K-12 strains were used as positive controls as it has previously been shown that fosmidomycin is active against these isolates [8].

2.2. Antimicrobial agents

Fosmidomycin was obtained from Invitrogen (Eugene, OR). All fosmidomycin derivatives were synthesised as previously described [9–13] (see Supplementary Fig. 1 for an overview of the structures).

2.3. Determination of minimum inhibitory concentrations (MICs)

MICs were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard broth microdilution protocol in Mueller–Hinton broth (MHB) [14]. A two-fold antibiotic dilution series ranging from 1 mg/L to 512 mg/L was tested. MICs were also determined in the presence of glucose-6-phosphate and glucose-1-phosphate as the sole carbon sources. To this end, a chemically defined minimal salt medium supplemented with 50 μ M FeCl₃.6H₂O (designated as CDM) [15] and equimolar concentrations of glucose-6-phosphate and glucose-1-phosphate instead of glucose (designated as CDM-G) was used.

2.4. RNA extraction and cDNA synthesis

Cells were cultured in either CDM or CDM-G medium. Antibiotic concentrations corresponding to the previously determined MIC values were used during treatment. If MIC values were >512 mg/L, cultures were grown in medium containing 512 mg/L

of the antimicrobial agent. After 24 h of incubation with constant agitation, cells were collected and RNA was extracted using a RiboPure™ Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions, except for the DNase treatment that was prolonged to 1 h. After RNA extraction, RNA yields were assessed using an RNA Quant-iT™ Kit (Invitrogen). Then, 15 µL RNA samples were used to synthesise cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

2.5. Quantitative real-time polymerase chain reaction (PCR)

Expression of seven genes (including two reference genes) was investigated by means of quantitative real-time PCR (qPCR). Primers were developed using the website of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) and their specificity was checked using BLAST (see Supplementary Table 1 for primer sequences). All qPCR experiments were performed on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler using iQ™ SYBR® Green Supermix Kit (Bio-Rad) according to the manufacturer's instructions. To activate the polymerase included in the iQ SYBR Green Supermix Kit, the reaction mixture was heated at 95 °C for 3 min, followed by 40 amplification cycles consisting of 15 s at 95 °C and 60 s at 60 °C. A melting curve analysis was included at the end of each run. Real-time PCR data were normalised to the geometric mean of the expression of two reference genes (BCAL0289 and BCAL1861), which were stably expressed in all conditions investigated in the present study (data not shown).

3. Results

3.1. Susceptibility testing in Mueller–Hinton broth

For all the BCC strains tested, MICs of fosmidomycin were >512 mg/L, whereas those for the two *E. coli* K-12 strains serving as positive controls ranged from 0.5 mg/L to 2 mg/L, in agreement with previously reported data [8].

The acetyl derivative FR900098 was capable of inhibiting the growth of 18 of the 40 strains tested at concentrations \leq 512 mg/L. *Burkholderia multivorans* LMG 18822 and LMG 18825, *B. cenocepacia* LMG 18830 and LMG 6986, *B. stabilis* LMG 14294^T, *B. vietnamiensis* LMG 18835 and LMG 10929^T, *B. dolosa* LMG 18941, *B. pyrrocinia* LMG 21824, *B. ubonensis* LMG 20358^T and LMG 24263, *B. seminalis* LMG 24067^T and *B. metallica* LMG 24068^T and R-2712 were inhibited at a concentration of 512 mg/L. Further, *B. cepacia* LMG 1222^T, *B. multivorans* LMG 13010^T and *B. lata* LMG 6992 were inhibited at a concentration of 256 mg/L and *B. anthina* LMG 20983 was inhibited at a concentration of 128 mg/L.

The seven other derivatives were tested against three BCC type strains, *B. cepacia* LMG 1222^T, *B. multivorans* LMG 13010^T and *B. cenocepacia* LMG 16656^T, but growth inhibitory concentrations of these derivatives were never <512 mg/L (Table 1).

3.2. Susceptibility testing in the presence of glucose-6-phosphate

First, the activity of fosmidomycin in the presence of glucose-6-phosphate was investigated by adding 25 mg/L glucose-6-phosphate to MHB. However, MICs remained >512 mg/L for all 40 strains tested (data not shown).

To investigate the activity of fosmidomycin in the presence of glucose-6-phosphate as the sole carbon source, CDM medium supplemented with Fe^{3+} [15] (designated CDM) and CDM with glucose-6-phosphate replacing glucose (designated as CDM + G6P) was used. Although it should be noted that reduced growth in these media precluded the determination of the exact MIC value, an increased sensitivity of *B. multivorans* LMG 13010^T and *B. cepacia* LMG 1222^T to fosmidomycin in CDM + G6P compared with CDM was observed (Fig. 1).

Because of the higher activity of FR900098, the effect of glucose-6-phosphate was further investigated using this acetyl derivative. MICs for *B. cenocepacia* LMG 6986, *B. multivorans* LMG 13010^T and *B. cepacia* LMG 1222^T were determined in CDM with glucose-6-phosphate and glucose-1-phosphate as the sole carbon sources (designated as CDM-G). Glucose-1-phosphate had no effect on MIC values but improved growth to levels equivalent to the growth in MHB (data not shown). MICs of FR900098 for all three strains tested were 64 mg/L in CDM-G compared with 512 mg/L for *B. cenocepacia* LMG 6986 and 256 mg/L for *B. multivorans* LMG 13010^T and *B. cepacia* LMG 1222^T in MHB.

3.3. Effect of glucose-6-phosphate on expression of genes involved in glycerol-3-phosphate transport during FR900098 treatment

Expression of three genes involved in glycerol-3-phosphate transport in *B. cenocepacia* LMG 16656^T (BCAL0282, BCAL0284 and BCAL0285) during treatment with 64 mg/L FR900098 was investigated. Expression levels for the three genes tested were significantly higher ($P < 0.05$) in the presence of glucose-6-phosphate (CDM-G) compared with controls (CDM) (Fig. 2a). Expression of BCAL0283, which encodes a putative ABC transporter permease involved in glycerol-3-phosphate transport, was too low to allow accurate quantification (data not shown).

3.4. Expression of BCAL2085 (*dxr*) and BCAL1451 (*fsr*)

Expression levels of *dxr* and *fsr* in the presence of fosmidomycin or FR900098 (64 mg/L) in CDM-G medium relative to untreated controls are shown in Fig. 2b. The *fsr* gene encoding an efflux pump was significantly upregulated during treatment compared with untreated controls ($P < 0.05$), whilst the gene encoding the target enzyme (Dxr) was stably expressed in both conditions.

4. Discussion

BCC bacteria are known for their resistance against a wide range of antimicrobial agents. The aim of the present study was to evaluate the potential of fosmidomycin for the treatment of BCC infections.

Despite the presence of *dxr* (BCAL2085) in the *B. cenocepacia* J2315 genome, a clear resistance to fosmidomycin and its derivatives was observed for all BCC strains

tested. This indicates that the presence of the non-mevalonate pathway, and more specifically Dxr, does not guarantee susceptibility towards inhibitors of this pathway and that resistance may be due to altered transport. The glycerol-3-phosphate transporter (GlpT) is reportedly the main importer of fosmidomycin in *E. coli* [6]. No *glpT* homologues were found in the *B. cenocepacia* J2315 genome, nevertheless an alternative system involved in glycerol-3-phosphate import is present. This transporter belongs to the ABC transporter superfamily and consists of a periplasmic substrate binding protein [*ugpB* (BCAL0282)], two permease proteins [*ugpA* (BCAL0283) and *ugpE* (BCAL0284)] and an ATP-binding protein [*ugpC* (BCAL0285)]. Because of the structural similarity of fosmidomycin to glycerol-3-phosphate, we suspect that fosmidomycin can enter *B. cenocepacia* cells through this alternative glycerol-3-phosphate transport system as well.

The activity of both fosmidomycin and FR900098 was enhanced in the presence of glucose-6-phosphate when used either as a sole carbon source or in combination with glucose-1-phosphate, suggesting the presence of an inducible sugar phosphate transport system through which fosmidomycin can be imported into BCC bacteria.

The hexose phosphate transporter (UhpT) is a main transport system for fosfomycin, a structural analogue of fosmidomycin, in *E. coli* cells [6,16]. Based on the genes involved in the hexose phosphate transport system in *E. coli*, we searched for their homologues in the genome of *B. cenocepacia* J2315 using BLAST. However, we did not identify *uhp* homologues, suggesting that *B. cenocepacia* lacks a specific sugar phosphate transport system.

This indicates that fosmidomycin can only be imported into *B. cenocepacia* cells through its glycerol-3-phosphate transport system. Therefore, the effect of glucose-6-phosphate on the expression of *ugpB*, *ugpC* and *ugpE* was investigated. A significant upregulation of these genes in CDM-G medium compared with CDM medium was observed. As the only difference between these media is the carbon source, the upregulation must be attributed to the presence of glucose-6-phosphate or glucose-1-phosphate in CDM-G medium. As previous observations indicated that glucose-1-phosphate had no influence on MIC values, the upregulation is likely caused by glucose-6-phosphate.

A gene conferring resistance to fosmidomycin (*fsr*) was shown to be involved in fosmidomycin efflux in *E. coli* [7]. As an *fsr* homologue is present in the genome of *B. cenocepacia* J2315 and is significantly upregulated in treated samples, this could in part explain the observed resistance in BCC bacteria.

In conclusion, these results suggest that the only transport system for fosmidomycin in BCC bacteria is a glycerol-3-phosphate transport system, which is induced in the presence of glucose-6-phosphate. Consequently, uptake of fosmidomycin can be increased by addition of glucose-6-phosphate. However, a fosmidomycin efflux pump is capable of compensating for this increased fosmidomycin uptake, providing resistance in BCC bacteria to fosmidomycin and FR900098.

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Competing interests

None declared.

Ethical approval

Not required.

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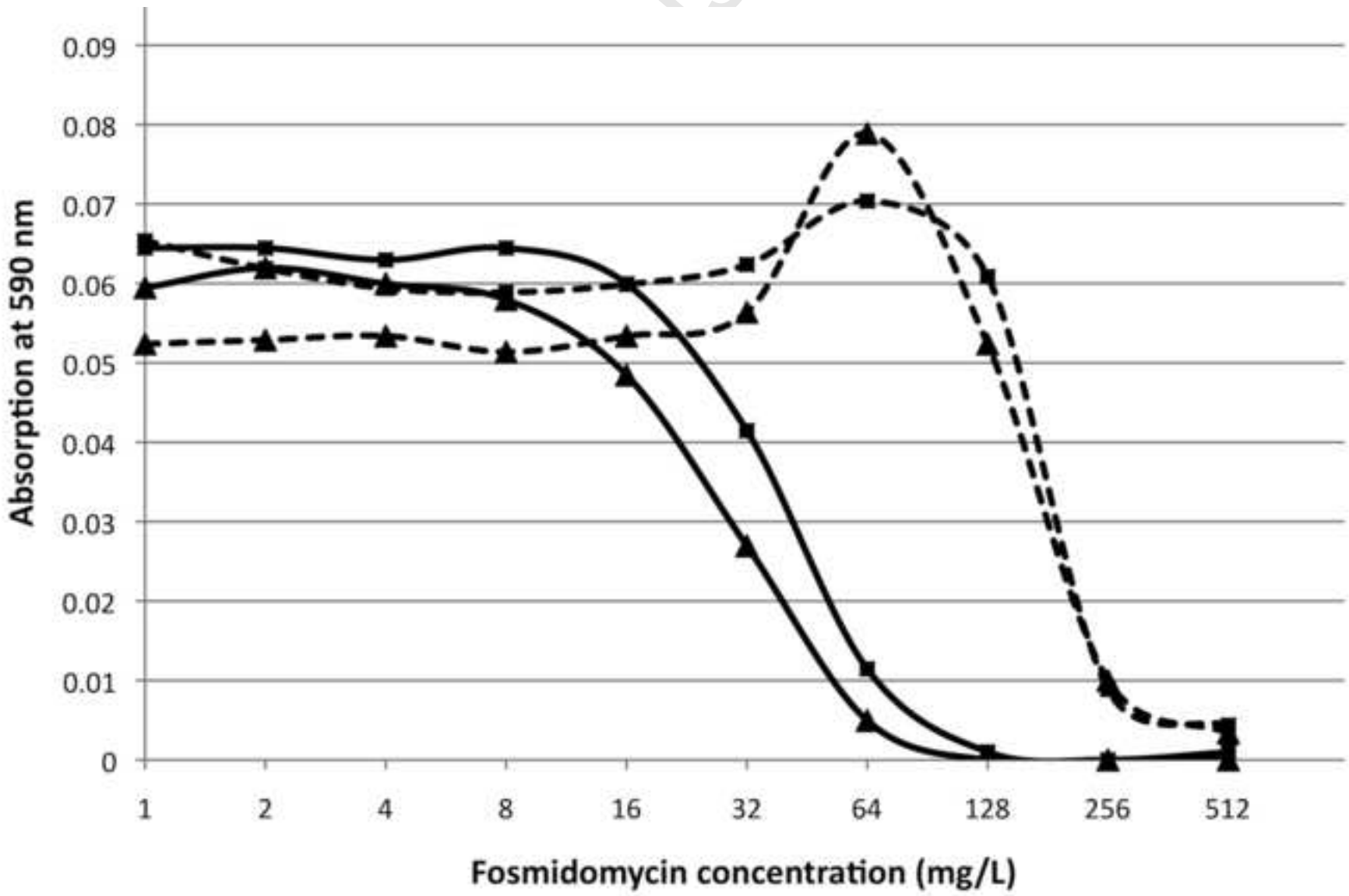
Fig. 1. Activity of fosmidomycin in the presence of glucose-6-phosphate as the sole carbon source. ---, activity in CDM medium; —, activity in CDM medium with glucose-6-phosphate replacing glucose; ■, *Burkholderia multivorans* LMG 13010^T; ▲, *Burkholderia cepacia* LMG 1222^T.

Fig. 2. (a) Transcriptional response of genes belonging to the glycerol-3-phosphate transport system to the addition of glucose-6-phosphate and FR900098 (expressed as fold change in CDM-G compared with CDM). (b) Expression of BCAL2085 (*dxr*) and BCAL1451 (*fsr*) in the presence of fosmidomycin and FR900098 (64 mg/L) (expressed as fold change in CDM-G with fosmidomycin or FR900098 compared with CDM). Experiments were performed in triplicate. CDM-G, CDM medium with equimolar concentrations of glucose-6-phosphate and glucose-1-phosphate instead of glucose.

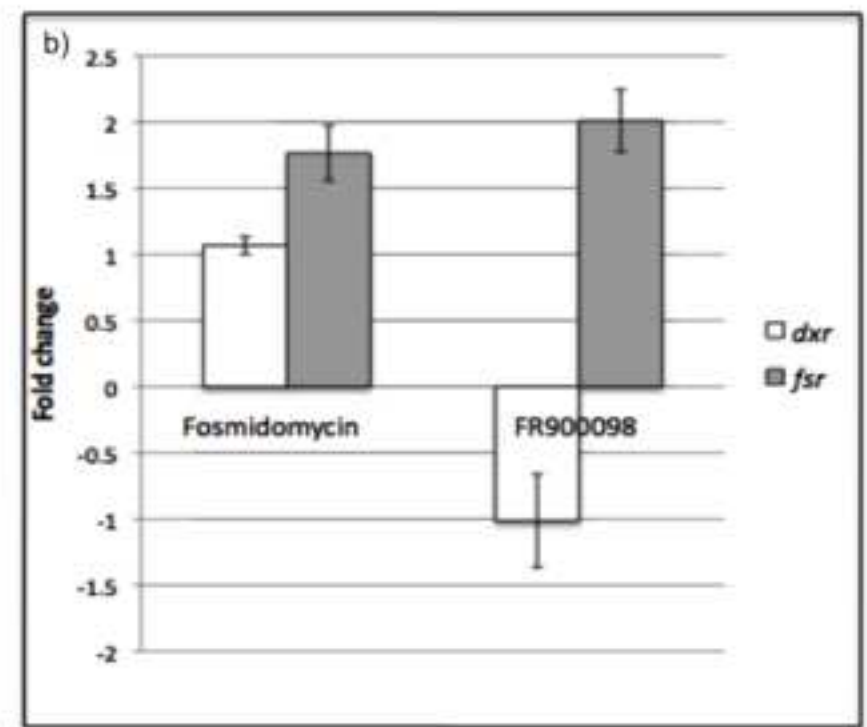
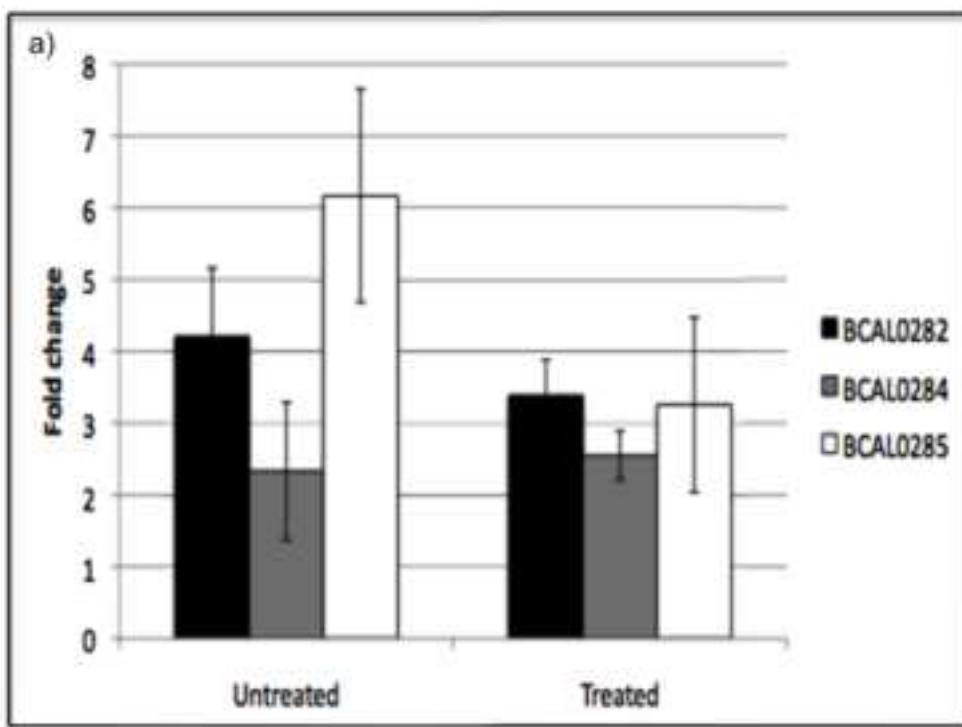
Appendix A: Supplementary data

Supplementary Fig. 1. Structure of fosmidomycin and the fosmidomycin derivatives studied.

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Supplementary Table 1

Forward (FW) and reverse (RV) primers used in real-time polymerase chain reaction (PCR)

Gene	Annotation	Orientation	Primer sequence (5'→3')	Concentration (nM)
BCAL2085	Dxr	FW	GCGCTCGAGGAAGGCGGTAT	600
		RV	TCCGGCGCTCGAGAAATGCT	600
BCAL1451	Efflux pump	FW	TGTTCCAGGTGGGCGGCAAT	300
		RV	CCAGTGGCCGATCTGCGTGA	300
BCAL0282	G3P transporter	FW	ATGCGAAGACGGGCCACCTC	200
		RV	GCCCGCCTTCTTGAACGCATC	200
BCAL0284	G3P transporter membrane protein	FW	GGGCTTCGACCTGTTCTGCCA	200
		RV	ACGCGACGTAGACGGGGAAC	200
BCAL0285	G3P transporter ATP-binding subunit	FW	CTGAGCTTGAAGGGCGTCAGGA	200
		RV	GGCCCGACCAGCACGATGAA	200
BCAL0289	Glutamate synthase	FW	ATCATCCAGCAGGGTCTGAAGA	600
		RV	GCCATTTCTCGCGATAGAA	600
BCAL1861	Acetoacetyl-CoA reductase	FW	GATCACCTGCTTCGTGACGTT	600
		RV	GACGTCGTGTTCCGCAAGAT	600

G3P, glycerol-3-phosphate.