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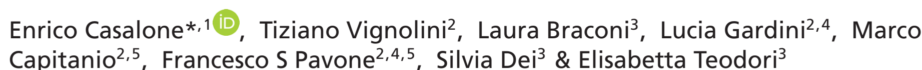
1-benzyl-1,4-diazepane reduces the efflux of resistance-nodulation-cell division pumps in *Escherichia coli*



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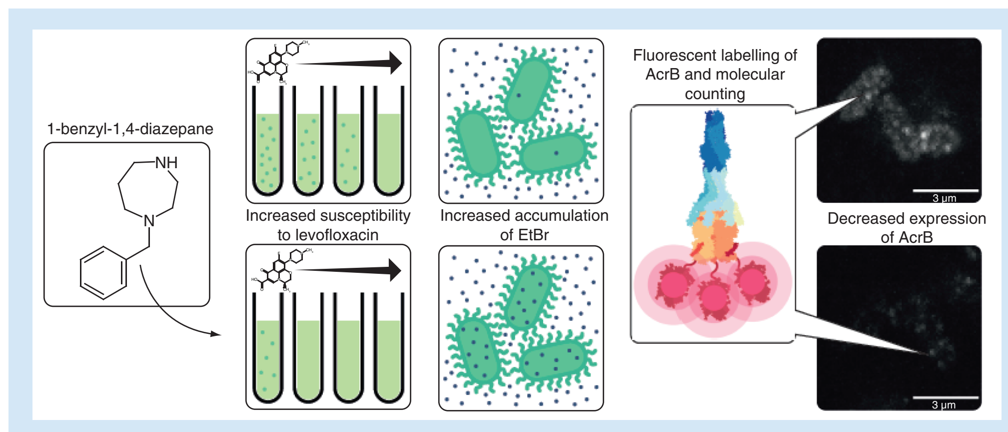
Aim: To investigate the action mechanism of 1-benzyl-1,4-diazepane (1-BD) as efflux pump inhibitor (EPI) in *Escherichia coli* mutants: Δ*acrAB* or overexpressing AcrAB and AcrEF efflux pumps. **Materials & methods:** Effect of 1-BD on: antibiotic potentiation, by microdilution method; membrane functionality, by fluorimetric assays; ethidium bromide accumulation, by fluorometric real-time efflux assay; AcrB expression, by quantitative photoactivated localization microscopy. **Results:** 1-BD decreases the minimal inhibitory concentration of levofloxacin and other antibiotics and increase ethidium bromide accumulation in *E. coli* overexpressing efflux pumps but not in the Δ*acrAB* strain. 1-BD increases membranes permeability, without sensibly affecting inner membrane polarity and decreases *acrAB* transcription. **Conclusion:** 1-BD acts as an EPI in *E. coli* with a mixed mechanism, different from that of major reference EPIs.

Graphical abstract:



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Keywords: AcrAB expression • *E. coli* • efflux-pump inhibitor • EPI • levofloxacin potentiation • MDR • membrane permeability • multidrug resistance

The large and often incorrect use of antibiotics has exerted selective pressure on susceptible bacteria, favoring resistant strains over time. One of the most successful strategies the bacteria use to adapt to chemically unrelated antimicrobial agents relies on the activation of ubiquitous efflux pumps (EPs) to extrude the drug [1–3]. In Gram-negative bacteria (GNB), the increased expression of EP genes acts synergistically with the outer membrane (OM),

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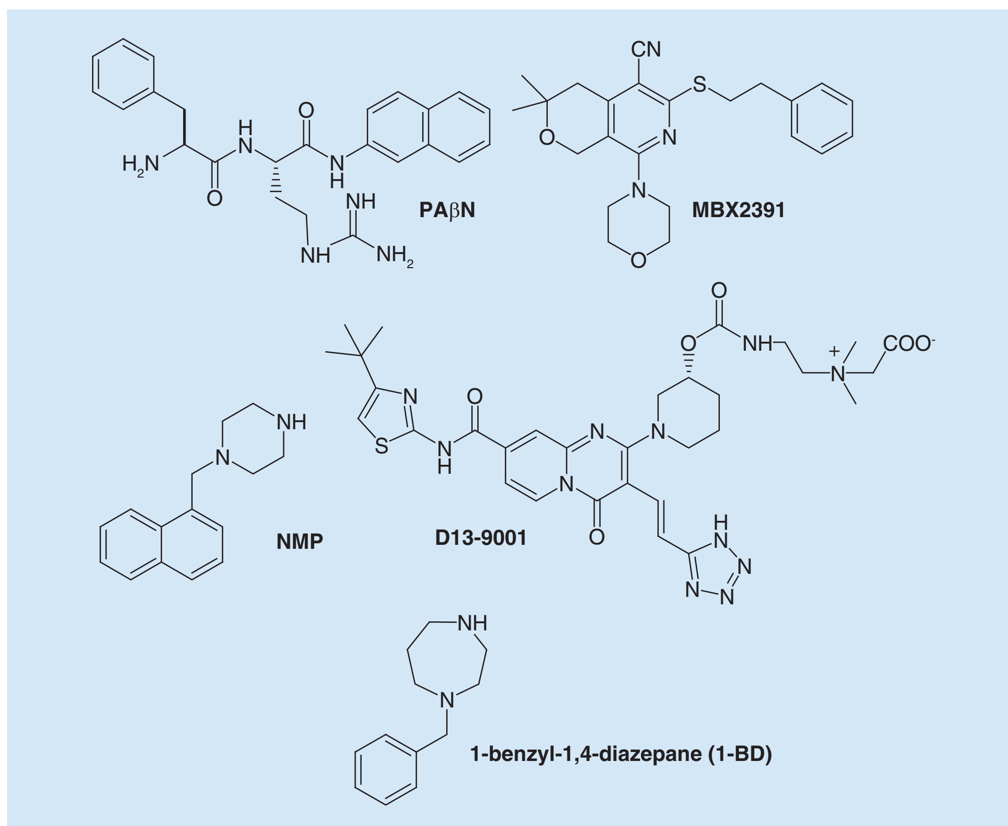

Figure 1. Structures of the main inhibitors of *Escherichia coli* resistance-nodulation-cell division efflux pumps and of 1-benzyl-1,4-diazepane.

resulting in a multidrug-resistant (MDR) phenotype [1,4]. AcrAB, belonging to the so-called resistance-nodulation-cell division (RND) family of bacterial EPs [1], is the main representative of the MDR-EPs in *Escherichia coli* [1,5]. AcrAB is predominant in GNB and associated with MDR in most clinically significant strains [1]. AcrAB–TolC is a tripartite complex (AcrA, AcrB and TolC), spanning across the inner membrane (IM) and OM of GNB [6,7] and can drive the extrusion of antibiotics directly from the cytoplasm into the external medium. RND pumps use proton motive force (PMF) as a source of energy and the IM protein AcrB catalyzes drug/H^+ antiport, in addition to being responsible for drug selectivity [7]. Deletions in *acrAB* or *tolC* genes resulted in increased susceptibility to a wide range of antibiotics and other toxic compounds [8]. Other RND EPs, like AcrEF and AcrAD, are also present in *E. coli* [9].

In EP-dependent MDR bacteria, the resistant phenotype may be reversed by using efflux pump inhibitors (EPIs), which act by increasing the intracellular concentration of the antibiotics [10]. The utilization of EPIs as adjuvants in antibiotic therapies is potentially of great interest in the battle against MDR pathogens [10,11]. Several natural and synthetic compounds are capable of inhibiting RND EPs in *E. coli* and other GNB [12]. Phenylalanine-arginine β-naphthylamide (PAβN), a synthetic dipeptide amine, MBX2391, a pyranopyrimidine, 1-(1-naphthylmethyl)piperazine (NMP), a piperazine derivative and the pyridopyrimidine D13-9001, are among the best characterized EPIs (Figure 1). Existing non-antibacterial drugs also demonstrated inhibitory activity by a more or less direct interaction with AcrAB–TolC [12]. However, problems like toxicity to humans, low water solubility and high affinity to serum albumin do not permit to any EPI to reach a clinical stage.

Expression of AcrAB in Enterobacteriaceae is subject to multiple levels of regulation [13]. In *E. coli* the expression is primarily controlled by the multiple antibiotic resistance (*mar*) operon by the global transcriptional activator MarA. MarA induces *acrAB* and *tolC* transcription and, increasing drug efflux, determined an MDR phenotype. MarR, another product of Mar operon, represses *marA* transcription. MarR has a strong affinity for aromatic molecules and MarA repression can be removed by phenolic compounds such as sodium salicylate [13] or diazepam,

Table 1. Genotype and pump related phenotype of the *Escherichia coli* strains used in this work.

Strain	Genotype				Pump-related phenotype
	gyrA	*mar*	*acrR* or *acrAB*	*acrS* or *acrEF*	
DC14PS	wt	wt	Inactivated[†]	wt	Not expressing AcrAB
1-DC14PS	S83L[‡]	wt	Inactivated	wt	Not expressing AcrAB
2-DC14PS	S83L	wt	Inactivated	IS2 inserted	Overexpressing AcrEF not expressing AcrAB
3-AG100MKX	S83L	Inactivated	IS186 inserted	wt	OverexpressingAcrAB
TV001	wt	wt	Ω*acrB::PAmCherry*	wt	Expressing AcrAB–PamCherry

[†] Gene inactivation in all strains was always obtained by insertion of a Kan^R cassette.
[‡] Single mutation.
wt: Wild-type.

a benzodiazepine sedative drug [14,15] in *E. coli*, resulting in a MDR phenotype. The local repressor AcrR acts as a modulator to prevent the overexpression of *acrAB* [13].

During a screening of a number of piperazines, aimed to the identification of putative EPIs, we found that the homolog 1-benzyl-1,4-diazepane (1-BD), also called 1-benzyl-1,4-homopiperazine (Figure 1), had some effect in reversing levofloxacin (LEV) resistance in *E. coli* mutants overexpressing RND EPs. The aim of this paper is to characterize the mechanism of action of 1-BD in the inhibition of the main RND EPs in *E. coli*.

Materials & methods

Chemicals

Levofloxacin, chloramphenicol (CHL), tetracycline (TET), rifampicin (RIF) and ethidium bromide (EtBr) were from Sigma-Aldrich Inc (MO, USA). NMP was from FluoroChem (Hadfield, UK), it was used as reference EPI [16]. 1-BD was a high-purity commercial compound from Sigma-Aldrich (Milan, Italy). All these molecules were dissolved in water. 1-BD showed a good water solubility and was dissolved in this solvent at a concentration of 13.3 mg/ml (0.07 M approximately).

Bacterial strains & growth conditions

All the strains used in this study are listed in Table 1. The AcrAB pump-deficient and the AcrAB and AcrEF overexpressing *E. coli* strains are derivatives of the AG100 *E. coli* strain [17]. All these strains carried a Kan^R cassette, which allowed us to select them on Luria Bertani (LB) agar in the presence of 100 μg/ml of kanamycin, before using the cells in subsequent assays. *E. coli* strains were grown in LB at 37°C. The *E. coli* strain TV001 used for the photoactivated localization microscopy (PALM) experiments is a derivative of the *E. coli* K12 strain BW25113 [18]. TV001 (Ω*acrB::PAmCherry*) was engineered by creating a fusion between the chromosomal *acrB* gene of BW25113 and a sequence encoding for PAmCherry, a photoactivatable fluorescent protein [19], through CRISPR/Cas9-assisted recombineering [20]. As such, all instances of AcrB expressed by TV001 are fused with a PAmCherry moiety [21]. Both BW25113 and TV001 were grown in LB with no added antibiotic.

Bacterial susceptibility testing

Susceptibility tests were performed by a twofold standard microdilution method following the Clinical and Laboratory Standards Institute recommendations [22]. Overnight cultures of *E. coli* were suitably diluted and inoculated (50 μl) in a 96-well Microtiter® plates to obtain approximately 5×10^5 CFU/ml in a final volume of 150 μl/well in the presence and absence (control) of different compounds. Minimum inhibitory concentration (MIC) values were detected in an Infinite M200 PRO Tecan microplate reader (Tecan® France, SA-Lyon, France) by reading optical density (OD) at wavelength 600 nm ($OD_{600\ nm}$) after 24 h incubation. The adjuvant-like effect of subtoxic concentrations (≤1/4 MIC) of 1-BD with LEV, CHL, TET and RIF were evaluated by three independent experiments carried out in duplicate. The results were expressed by the activity gain parameter (A): $A = MIC_{ant}/MIC_{ant+1\text{-}BD}$, where MIC_{ant} is the MIC value of the antibiotic alone and $MIC_{ant+1\text{-}BD}$ the MIC of antibiotic in the presence of 1-BD; the higher the A value, the greater the adjuvant effect of 1-BD toward the antibiotic. Checkerboard assay was performed in duplicate, essentially as described previously [23], on the 1-DC14PS, 2-DC14PS and 3-AG100MKX strains by assembling a 2D array of serial dilutions of 1-BD (3.2–0.05 mg/ml) and LEV (16–0.125 μg/ml) in a 96-well Microtiter plate. The interaction between 1-BD and LEV was evaluated by the fractional inhibitory

concentration (FIC) index (FICi): FICi = FIC_{LEV} + $FIC_{1\text{-}BD}$, where FIC_{LEV} = $MIC_{LEV + 1\text{-}BD}/MIC_{LEV}$ and $FIC_{1\text{-}BD}$ = $MIC_{1\text{-}BD + LEV}/MIC_{1\text{-}BD}$. Considering the FICi values, the nature of the interaction was as follow: ≤0.5, synergy; >0.5–1, additive; >1 to <2, indifference; ≥2 antagonism [23]. MIC values are geometric means of the replicate assays; the level of sample reproducibility was always within one-doubling-dilution difference that is satisfying in MIC testing.

EtBr accumulation by fluorometric real-time efflux assay

EtBr, a fluorescent substrate of RND pumps that diffuses passively through the bacterial membrane and becomes strongly fluorescent after its irreversible binding to nucleic acids, has been used as a probe to evaluate the efflux activity of bacterial cells [24]. The assay was performed in a black flat bottom 96-well microplate, at equilibrium concentration of EtBr ($[EtBr]_{eq}$), at which influx equals efflux [25]. To determine the $[EtBr]_{eq}$, a cell suspension with $OD_{600\ nm}$ of 0.6 in a final volume of 150 μl phosphate-buffered saline (pH 7.4) with 0.4 % glucose was exposed for 60 min at 37°C, in the absence of 1-BD, at increasing concentration of EtBr (from 0.0625 to 6 μg/ml). The choice of this range of EtBr concentrations was based on data reported in literature about the EtBr sensitivity of *E. coli* AG100 strains isogenic to our ones [25]. Concentrations of EtBr up to 5 μg/ml did not affect the growth of an *E. coli* AG100 mutant not expressing AcrAB while 300 μg/ml of EtBr are needed to inhibit the growth of a AG100 mutant overexpressing AcrAB [25]. The microplate was read at intervals of 60 sec with the Tecan infinite M200 PRO reader, excitation and detection wavelength of fluorescence were 530 nm and 585 nm, respectively.

The effect of 1-BD on the accumulation of EtBr was then evaluated at $[EtBr]_{eq}$, in *E. coli* cells treated with 0.8 (1/2 MIC) or 0.4 mg/ml 1-BD (1/4 MIC); untreated cells were used as control. The accumulation of EtBr was reported as the relative final fluorescence (RFF) index: RFF = $(RF_{treated}\text{-}RF_{untreted})/RF_{untreated}$. An RFF index >1 indicates an evident increased accumulation of EtBr compared with the control (non-treated cells taken as 0). All experiments were done in duplicate. A Student's *t*-test has been applied to evaluate statistically significant differences.

Membranes permeability assay

The effect of 1-BD on membranes integrity was evaluated using the Live/Dead BacLight Bacterial Viability Kit (Life Technology, MA, USA). *E. coli* strains were grown at 37°C with shaking until $OD_{600\ nm}$ of 0.12. Aliquots of 0.5 ml of *E. coli* cultures were incubated 1 h at 25°C with 1-BD concentrations from 0.2 to 0.8 mg/ml. Bacterial cells were centrifuged (10,000×*g* for 10 min a 5°C) and resuspended in 0.5 ml of a saline solution. 100 μl of cell suspension were transferred into a black flat 96-well microtiter plate, where 50 μl of the SYTO 9/propidium iodide dyes (ratio 1:1) were added to each well and the final volume adjusted to 200 μl with distilled water. The microplate was incubated 15 min at room temperature in the dark. A Tecan infinite M200 PRO microplate reader set at fluorescence excitation wavelength of 485 nm, was used to measure the fluorescence of SYTO 9 (green) and propidium iodide (red) at emission wavelength of 528 nm and 590 nm, respectively. Bacteria with intact cell membranes fluoresced green and bacteria with compromised membranes fluoresced red. The green/red ratios were determined and the results were reported as the percentages of intact membranes in treated cells compared with the untreated control. All experiments were done in duplicate. A Student's *t*-test has been applied to evaluate statistically significant differences.

Membrane potential assay

The effect of 1-BD on membrane PMF was measured using the BacLight Bacterial Membrane Potential Kit (Life Technology). 1-BD was serially diluted 1:2 in sterile distilled water from 3.2 to 0.4 mg/ml into black flat bottom 96-well plates, and 5×10^5 cell/ml *E. coli* cells from fresh cultures and 30 μM of the green fluorescent 3,3′-diethyloxacarbocyanine iodide were added to each sample to reach a final volume of 150 μl; the plates were incubated statically for 30 min at room temperature in the dark. Negative (water instead of 1-BD) and positive (78 μM carbonyl cyanide-m-chlorophenylhydrazone, to dissipate the PMF) controls were also present. 3,3′-Diethyloxacarbocyanine iodide fluoresces green, but the fluorescence shifts toward red when it is in a self-associated form, as it happens when the cell depolarizes and its cytosolic concentration increases. A microplate reader Tecan infinite M200 PRO, set at excitation and detection wavelength of 528 nm (green) and 590 nm (red), respectively, was used to measure fluorescence. The red–green ratio was determined and expressed as percentage of depolarized membranes by normalizing against the emission from the negative control. All experiments were done in duplicate. A Student's *t*-test has been applied to evaluate statistically significant differences.

Table 2. Adjuvant effect, of 1/4 minimum inhibitory concentration of 1-benzyl-1,4-diazepane (0.4 mg/ml) and 1-(1-naphthylmethyl)piperazine (0.1 mg/ml), expressed as gain in antibiotic activity (A), in 1-DC14PS, 2-DC14PS and 3-AG100MKX *Escherichia coli* strains.

Strain	Antibiotic	MIC (μg/ml)			Antibiotic activity gain (A)	
		No EPI	1-BD	NMP	1-BD	NMP
1-DC14PS	LEV	0.25	0.25	0.25	1	1
	CHL	2	2	2	1	1
	TET	4	2	2	2	2
	RIF	32	32	4	1	8
2-DC14PS	LEV	4	0.5	0.25	8	16
	CHL	8	2	2	4	4
	TET	8	4	2	2	4
	RIF	8	32	8	0.25	1
3-AG100MKX	LEV	4	1	0.5	4	8
	CHL	8	4	8	2	1
	TET	8	4	4	2	2
	RIF	32	32	8	1	4

1-BD: 1-Benzyl-1,4-diazepane; CHL: Chloramphenicol; EPI: Efflux pump inhibitor; LEV: Levofloxacin; MIC: Minimum inhibitory concentration; NMP: 1-(1-Naphthylmethyl)piperazine; RIF: Rifampicin; TET: Tetracycline.

Quantitative PALM experiments

1-BD was characterized in its effect on the expression level of AcrB with quantitative super-resolution fluorescence microscopy. *E. coli* strain TV001 was grown at 37°C with shaking (210 r.p.m.) overnight. Afterward, the cells were diluted 1:100 in fresh LB medium and kept at 37°C with shaking in vented tubes for 2 h to reach the logarithmic growth phase. After 2 h, bacteria were treated with 1-BD at 0.4 mg/ml final concentration or sodium salicylate at 0.8 mg/ml final concentration. A third sample was left untreated as a control. Bacteria were exposed to treatments for 2 h while shaking at 37°C. After two washing steps by centrifuging at 5000×*g* for 5 min at room temperature and resuspending in phosphate-buffered saline (PBS), cells were fixed by resuspending the pellet in 0.5 ml of a 4% paraformaldehyde solution in PBS and incubating them at room temperature for 10 min. After fixation, the samples were washed twice by centrifuging and resuspending the pellet in 1 ml of PBS. The samples were stored at 4°C for less than 24 h prior to imaging. Imaging chambers were built by placing microscope slides on top of poly-L-lysine-coated coverslips, separating them with thin strips of double sticky tape [26]. The bacterial suspensions were mounted for imaging by fluxing them within the chambers and letting them adhere to the poly-L-lysine-coated coverslips for at least 20 min.

Imaging was performed on a custom-built widefield fluorescence microscope with single-molecule detection capabilities [26–28]. PALM was executed by activating PAmCherry with 405 nm light and recording the fluorescence signal emitted by single fluorescent proteins under 532 nm excitation until complete photobleaching of PAmCherry was observed. Acquired videos were analyzed with the ThunderSTORM ImageJ plugin [29] in order to reconstruct super-resolved molecular maps. Assignment of localized molecules to individual bacteria was then performed through a custom-made Matlab pipeline (Vignolini *et al.*, Manuscript in Prep.). All experiments were done in triplicate. A Student's *t*-test has been applied to evaluate statistically significant differences.

Results

Antimicrobial activity of 1-BD, NMP & antibiotics in *E. coli* strains overproducing or lacking major EPs of the RND type

MICs of 1-BD and NMP against different *E. coli* strains are reported in Supplementary Table 1. 1-BD was less toxic compared with NMP. $MIC_{1\text{-}BD}$ was 1.6 mg/ml in both pump-deficient and pump-overexpressing strains; similarly, MIC_{NMP} was the same in all the tested strains, even though lower (0.4 mg/ml) than $MIC_{1\text{-}BD}$.

Table 2 reports the MICs of different antibiotics in the same *E. coli* strains. As expected, antibiotics that are substrates of AcrAB and AcrEF pumps, LEV, CHL and TET had MICs that were 16, 4 and 2, respectively, times higher in the overexpressing strains 2-DC14PS and 3-AG100MKX compared with 1-DC14PS strain. Whereas MIC values of RIF, an antibiotic that is considered a rather poor substrate of *E. coli* RND type EPs [30], were higher

Table 3. Accumulation of ethidium bromide in *Escherichia coli*, expressed as relative final fluorescence (RFF).

Strain	1-BD (mg/ml)	EtBr (μg/ml)	
		6	0.5
DC14PS	0.8	–	0.70 ± 0.56
	0.4	–	0.33 ± 0.10†
1-DC14PS	0.8	–	1.07 ± 0.61
	0.4	–	0.60 ± 0.01
3-AG100MKX	0.8	3.66 ± 2.18	–
	0.4	2.00 ± 0.50†	–
2-DC14PS	0.8	3.92 ± 0.03	–
	0.4	2.49 ± 1.34	–

Note: RFF values at [EtBr]eq in *E. coli* strains DC14PS and 1-DC14PS (0.5 μg/ml of EtBr), 3-AG100MKX and 2-DC14PS (6 μg/ml) in the presence of subtoxic concentrations of 1-benzyl-1,4-diazepane. The data represent mean values ± standard deviation from two independent experiments.
† RFF values with statistically significant difference ($p < 0.05$) according to Student's *t*-test.
1-BD: 1-Benzyl-1,4-diazepane; EtBr: Ethidium bromide; [EtBr]eq: Equilibrium concentration of EtBr; RFF: Relative final fluorescence.

and similar in 1-DC14PS and 3-AG100MKX, respectively, but four times lower in 2-DC14PS, suggesting that RIF is anyway a better substrate for AcrAB than for AcrEF.

1-BD potentiates the antibacterial activity of different antibiotic against *E. coli*

As reported in Table 2, in the presence of 0.4 mg/ml 1-BD (1/4 MIC), a potentiated activity (A >1) has been highlighted in 2-DC14PS and 3-AG100MKX for LEV (A = 8 and A = 4, respectively) and CHL (A = 4 and A = 2), but not in 1-DC14PS (A = 1 for both LEV and CHL), whereas for TET a potentiated effect (A = 2) was detected in all strains. As expected for an antibiotic that is a poor substrate of RND pumps, 1-BD does not enhance the effect of RIF (A ≤ 1) in any strain, rather it appears to depower RIF in 2-DC14PS (A = 0.25), by what mechanism it is not easy to imagine. The reference EPI NMP at 1/4 MIC (0.1 mg/ml) acted similarly but, in contradiction with previous results [16,30], it did not enhance the activity of CHL (A = 1) in 3-AG100MKX.

The effect of 1-BD on LEV activity from two independent checkerboard assays, whose results were perfectly matching, are reported in Supplementary Table 2. In 3-AG100MKX and 2-DC14PS, overexpressing AcrAB and AcrEF, respectively, 0.4 mg/ml of 1-BD determined a synergic effect (FICi ≤0.5) on LEV, whereas at lower concentration 1-BD showed an additive effect (FICi >0.5–1). In any case, indifference (FICi >1) characterized the 1-BD/LEV interaction in the 1-DC14PS strain lacking AcrAB.

1-BD inhibits the efflux of EtBr

The gain in antibiotic activity (A) and FICi consider bacterial growth inhibition to evaluate 1-BD effect on antibiotic efflux. A more direct measure of EPI activity of 1-BD was obtained by measuring the intracellular accumulation of EtBr by real-time fluorometric assay.

The accumulation kinetics of EtBr in the absence of 1-BD, in order to determine the $[EtBr]_{eq}$ in each *E. coli* strain, is reported in Supplementary Figure 1. As expected for a pump substrate, $[EtBr]_{eq}$ was found to be dependent on the EP expression. The AcrAB and AcrEF overexpressing mutants, 3-AG100MKX and 2-DC14PS respectively, had a $[EtBr]_{eq}$ higher (6 μg/ml) than the AcrAB-deficient mutants DC14PS and 1-DC14PS (0.5 μg/ml).

The addition of 1-BD at $[EtBr]_{eq}$ resulted in a concentration-dependent increase in relative fluorescence intensity in both AcrAB-deficient and AcrAB-overexpressing strains (Figure 2). However, while in the deficient ones the increase was very low, in the overexpressing ones the EtBr accumulation was much higher, immediately reaching high levels. A quantitative evaluation of the ability of 1-BD to promote the intracellular accumulation of EtBr is reported in Table 3 as RFF data, where relative is referred to the control without 1-BD. The EtBr accumulation results were always higher at 1-BD concentration of 0.8 mg/ml than at 0.4 mg/ml. Compared with controls without molecule, 1-BD at 0.4 mg/ml (1/4 MIC), determines intracellular accumulation of EtBr in the EP overexpressing mutants (RFF >1), but not in the *acrAB*-deleted mutants (RFF <1). Similar results were also reported for NMP [16]. A significant difference was observed only between 1-DC14PS and 3-AG100MKX at 0.4 mg/ml 1-BD. However, if the RFF values at 0.4 mg/ml 1-BD of the two overexpressing strains were aggregated and the mean compared with the mean of the corresponding aggregated values of the AcrAB-deficient strains, a significant difference ($p < 0.01$) was observed.

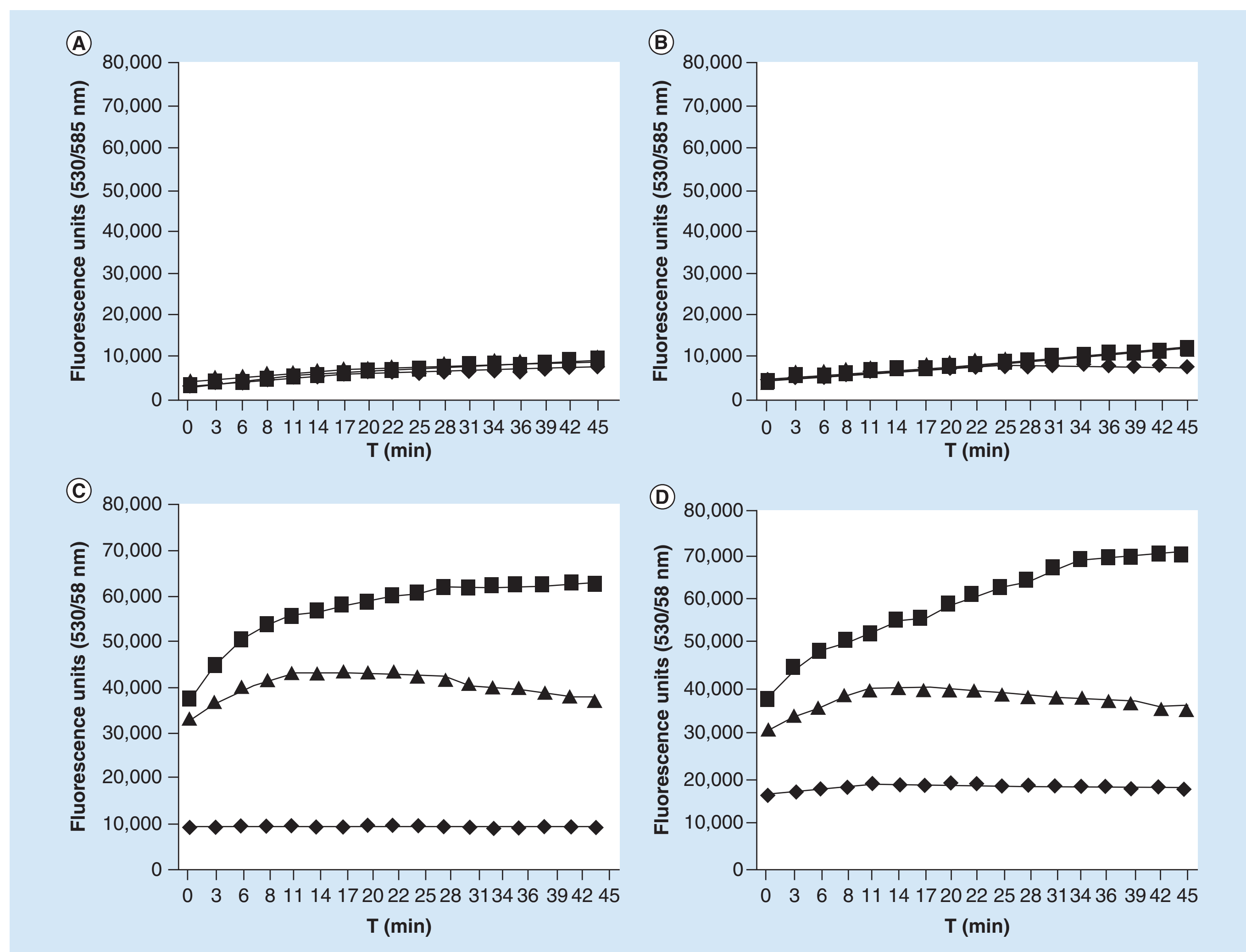

Figure 2. Effect of 1-BD on the accumulation of EtBr in *Escherichia coli*. (A) DC14PS; **(B)** 1-DC14PS; **(C)** 2-DC14PS and **(D)** 3-AG100MKX strains in the presence of $[EtBr]_{eq}$ (0.5 µg/ml in **A** and **B**; 6 µg/ml in **C** and **D**) and at different concentration of 1-BD (♦, no molecule; ▲, 0.4 mg/ml; ■, 0.8 mg/ml) at 37°C in the presence of glucose.
1-BD: 1-Benzyl-1,4-diazepane; EtBr: Ethidium bromide; [EtBr]eq: Equilibrium concentration of EtBr.

1-BD affects the integrity of the membranes

In Figure 3, the relative fluorescence green/red ratios of treated cells respect to untreated cells (green/red = 100%) are reported. 1-BD induced a concentration dependent decrease of the integrity of the membranes (decrease in green/red ratio) in the three strains tested. 1-BD at 0.4 mg/ml (1/4 MIC) caused integrity reduction of about 25 and 33 % in the pump overexpressing mutants 3-AG100MKX and 2-DC14PS, respectively, significantly lower than that measured in 1-DC14PS (about 57 %). The permeabilization effect increased at the increase of 1-BD concentration and the differences between the overexpressing strain and the AcrAB-deleted strains remained significant.

In all tested strains a modest IM depolarization effect by 1-BD was observed, even at the highest concentrations of the molecule (Figure 4). Surprisingly, at 1/4 $MIC_{1\text{-}BD}$, the depolarization in 3-AG100MKX (about 14%) was significantly higher than that measured in 1-DC14PS and 2-DC14PS (about 2% in both strains). No significant differences were observed at higher concentration of 1-BD.

1-BD decrease the expression of AcrB

To investigate a possible effect of 1-BD on the expression level of AcrB, we conducted quantitative single-molecule localization microscopy experiments on *E. coli* strain TV001, expressing a fluorescently-labeled version of AcrB

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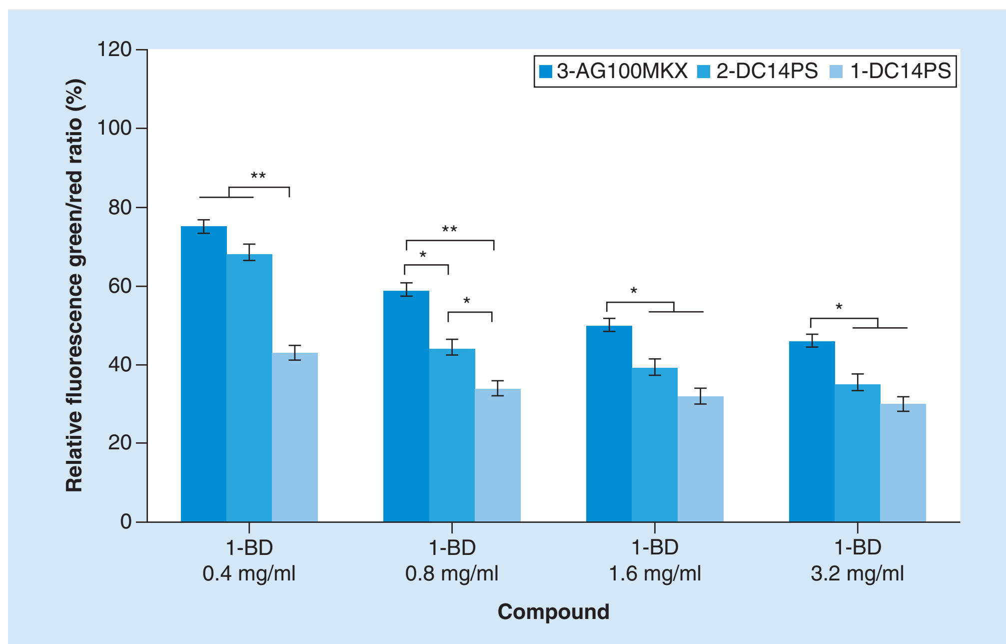

Figure 3. Effect of different concentrations of 1-benzyl-1,4-diazepane on the permeabilization of cell membranes in the *Escherichia coli* strains 3-AG100MKX, 2-DC14PS and 1-DC14PS. The values shown represent averages of two independent experiments and respective standard deviations. Asterisks indicate statistically significant differences according to Student's *t*-test.
*$p < 0.05$; **$p < 0.01$.

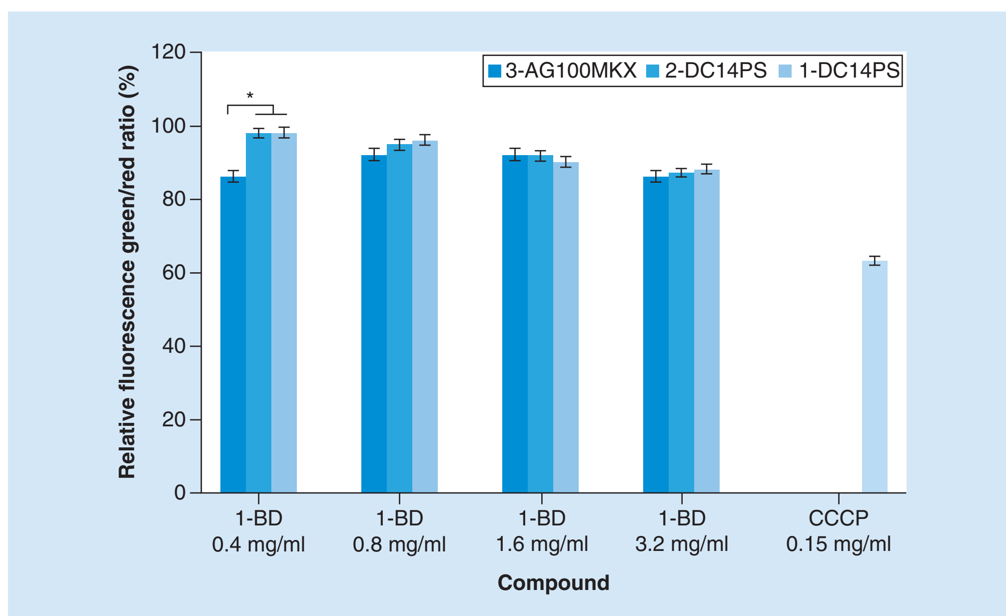

Figure 4. Effect of different concentrations of 1-benzyl-1,4-diazepane on inner membrane potential in the *Escherichia coli* strains 3-AG100MKX, 2-DC14PS and 1-DC14PS. The values shown represent averages of two independent experiments and respective standard deviations. CCCP was used as positive control for its ability to dissipate the proton gradient. Asterisks indicate statistically significant differences according to Student's *t*-test.
*$p < 0.05$.
CCCP: Cyanide-m-chlorophenylhydrazone.

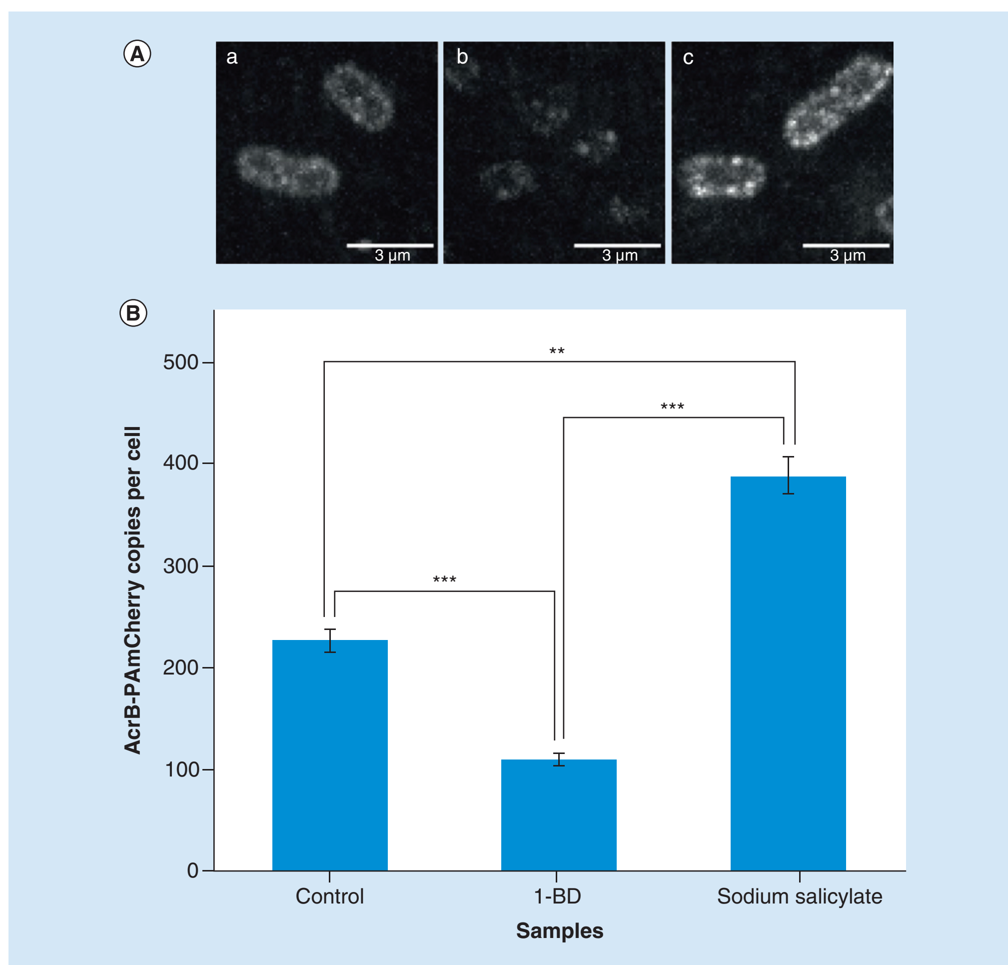

Figure 5. Effect of 1-benzyl-1,4-diazepane on the expression of AcrB in the *Escherichia coli* strain TV001 expressing AcrB fused to PAmCherry photoactivable fluorescent protein. (A) PALM images of *E. coli* TV001 cells after 2 h of incubation with either (a) plain LB medium (control), (b) 0.4 mg/ml 1-BD or (c) 0.8 mg/ml sodium salicylate. Each image is the result of a maximum intensity projection over 1000 consecutive frames. **(B)** Average number of AcrB–PAmCherry copies per bacterium after 2 h of incubation in control cells (n = 169), with 1-BD (n = 185) and sodium salicylate (n = 136) as a positive control. The values shown represent averages of three independent experiments and respective standard errors and were compared through Student's *t*-test *p < 0.05; **p < 0.01; ***p < 0.001.
1-BD: 1-Benzyl-1,4-diazepane; LB: Luria Bertani; PALM: Photoactivated localization microscopy.

from the original chromosomal locus. This technique allows for a direct measurement of the quantity of AcrB pump on the membrane of live bacteria under different conditions. In Figure 5, we report examples of the resulting fluorescence microscopy images of individual bacterial cells after 2 h of incubation with either 1-BD, sodium salicylate, or nothing (control). Sodium salicylate was used as a positive control for its ability to upregulate the expression of the *acrAB* operon by disrupting MarR-mediated repression [13,14]. Figure 5 also reports the average number of AcrB–PAmCherry copies observed by image analysis. There is a statistically significant decrease in the amount of observable AcrB–PAmCherry in bacterial cells after exposure to 1-BD (n = 185) with respect to unexposed controls (n = 169), which suggests an inhibitory effect of 1-BD on the expression of *acrAB*. The number of AcrB–PAmCherry copies in bacteria treated with 1-BD (110.1 ± 6.1) is about half of the copies measured in control cells (226.5 ± 11.2). On contrary, in the presence of sodium salicylate (n = 136), AcrB–PAmCherry expression is roughly doubled (389.2 ± 18.3).

Discussion

The lack of effective treatment against infections caused by MDR bacteria is a worldwide healthcare emergency. Overexpression of EPs extruding antibiotics is a major mechanism in the resistance of clinical isolates [2]. For their

essential role in removing antibiotics from bacterial cells increasing antibiotic resistance, EPs are very interesting target of combined, EPI plus antibiotic, therapies; an approach aimed to restore the effectiveness of the antibiotic in MDR bacterial pathogens. Developing clinically useful EPIs is a challenge; none of the so far identified EPIs have reached the clinical stage. This study reports the characterization of a novel EPI, 1-BD, able to inhibit major RND-type EPs of *E. coli*.

The ability of 1-BD, at subtoxic concentrations, to enhance the activity of LEV and other unrelated antibiotics (Table 2 & Supplementary Table 2) and to decrease EtBr extrusion in the 3-AG100MKX and 2-DC14PS *E. coli* strains overexpressing AcrAB and AcrEF, respectively, but not in the DC14PS and 1-DC14PS strains lacking AcrAB (Table 3), strongly indicates that 1-BD would act as an inhibitor of AcrAB and AcrEF efflux capacity in *E. coli*. However, since the MIC of 1-BD was not influenced by the level of expression of Acr AB and AcrEF (Supplementary Table 1), 1-BD looks like a poor substrate of AcrAB and AcrEF. Consequently, like NMP and D13-9001 [1,31] and unlike PAβN [1], it seems very unlikely that the ability of 1-BD to enhance the growth inhibitory activity of antibiotics is the result of competition with the antibiotic transport process by these pumps. 1-BD, independently from the expression levels of AcrAB and AcrEF, did not show any adjuvant effect on RIF (Table 2), an antibiotic that is not a substrate of these pumps; this behavior differentiates 1-BD from NMP (Table 2), and PAβN [16]. As so far discussed, the inhibiting effect of 1-BD on the efflux of LEV and EtBr in *E. coli* could be ascribable to a more or less direct and specific effect on AcrAB and AcrEF activity. Anyway, by the above reported data we could not have excluded that 1-BD can also act by affecting membranes functionality. And in fact, 1-BD determinates a noticeable, concentration-dependent, permeabilization effect on membranes in *E. coli* (Figure 3). The effect was significantly higher in the *acrAB* knockout strain 1-DC14PS than in the *E. coli* strains overexpressing AcrAB and AcrEF, suggesting that a larger number of RND pumps complexes can protect cellular membranes (IM mostly) from the destabilization effects of 1-BD. A membrane permeabilization effect was also reported for PAβN in *E. coli* [32,33] and *P. aeruginosa* [31], and for NMP in *Klebsiella pneumonia* [34], whereas D13-9001 did not permeabilized the IM in *E. coli* [33]. PAβN also affected the OM permeability in *Pseudomonas aeruginosa* with potency similar to polymyxin B nonapeptide, a compound well known for its permeabilizing activity on both IM and OM [31]. The contribution of the permeabilizing effect, by increasing antibiotics diffusion across membranes, must be considered in the evaluation of the adjuvant activity of 1-BD. The integrity of the IM is also essential for the preservation of the membrane potential, which supply the energy necessary to the operation of RND and other families of pumps.

1-BD showed little depolarizing effect on IM (Figure 4), such that the contribution of this effect to the observed enhancement of LEV activity and the accumulation of EtBr should also be very limited. A membrane potential reduction has been observed in *Klebsiella pneumoniae* exposed to NMP [34] and membrane depolarization is also the main mechanism for efflux-inhibition activity by the putative EPI 2-phenylquinolone (PQQ4R) in *E. coli* [32]. Even PAβN, not usually considered a proton conductor [1], has been reported to possess depolarizing activity in *E. coli* [33] and in *P. aeruginosa* [31].

For all of the above said, 1-BD appears to act as an antibiotic's enhancer by a mixed mechanism, which combines a noncompetitive inhibition of antibiotic transport by RND-type EPs to a membrane permeabilization activity. Everything considered, this mechanism seems different from those shown by the major EPIs NMP, PAβN, D13-9001 and PQQ4R. In addition to this dual mechanism of action, no doubt 1-BD also downregulates *acrB* expression, halving its presence on the IM of *E. coli* (Figures 5). The downregulation effect of 1-BD on *acrAB* could be underestimated, because partially masked by 1-BD effect on IM permeability. In fact, as observed in *Pseudomonas fluorescens*, IM permeabilization could be responsible of enhanced expression of RND-type EPs, in the effort to repair membrane damage by increasing fatty acid efflux [35]. We can only speculate on how 1-BD downregulates the expression acrAB. Since salicylate is able to detach MarR from the marbox, inducing MarA expression and consequently that of *acrAB* and *tolC* genes [36], in the opposite way, 1-BD could bind to MarR, stabilizing it on the marbox of operon Mar, repressing the expression of *acrAB* and *tolC* genes. However, as *marR* is disrupted in 3-AG100MKX strain, 1-BD could act downstream the expression cascade of *acrAB*. MarA or their homologues SoxS and Rob, involved in the transcription of *marRAB* and *AcrAB–TolC* [13] or AcrS/EnvR, known to represses the expression of *acrAB* and *acrEF*, in *E. coli* [37], could be possible target candidates of 1-BD. As far as we know, none of the best characterized putative EPIs (NMP, PAβN, MBX2391 and D13-9001) have been reported to act by inhibiting the expression of *acrAB* or other EP genes in bacteria. However, different molecules from plant extracts have been reported, by transcription analysis or by reporter genes, to downregulate the expression of EP genes of the ABC class [10] and of AcrAB–TolC in *E. coli* [38] and *Staphylococcus aureus* [39]. Diclorofenac, a nonsteroidal anti-inflammatory drug, also strongly downregulated antimicrobial EPs of *S. aureus* [40]. In this work

we used, for the first time, quantitative single-molecule localization microscopy to evaluate the effect of an EPI on the expression of an EP. This technique allows to look directly at the end product of the gene expression cascade, the AcrB protein, right where it is expected to be, in the IM.

1-BD showed a low cytotoxicity (in the mM range) in a preliminary MTS test on cultured primary Human Dermal Fibroblasts (data not shown). This result is encouraging because cytotoxicity is one of the major drawbacks to the clinical use of putative EPIs. For example, NMP and PAβN are toxic because of their serotonin agonist properties [41] and membrane depolarizing activity [42], respectively.

Conclusion

1-BD shows most of the ideal characteristics of an EPI: it has no relevant intrinsic antibacterial activity (high MIC values); at subtoxic concentrations (≤1/4 MIC), it enhances the activity of the antibiotics LEV, CHL, TET, substrates of EPs (but not that of the non-substrate RIF) in strains overexpressing EPs (3-AG100MKX and 2-DC14PS), but not in strains lacking AcrAB (DC14PS and 1-DC14PS); it decreases the extrusion of EtBr by AcrAB and AcrEF in 3-AG100MKX and 2-DC14PS, but not in 1-DC14PS; it has noticeable effects on membranes integrity but very little on proton gradient across the IM. Moreover, 1-BD downregulates *acrB* expression in *E. coli*. 1-BD seems to act by a mixed mechanism, overall different from that of so far quite well characterized EPIs. However, the knowledge of the large number of different EPs and the complex regulation of their expression in *E. coli* [1,11] suggest caution in the interpretation of the data we report. Further investigation will be necessary for a more comprehensive view of the mechanism of action of 1-BD as EPI. Thanks to low cytotoxicity and good solubility in water, and despite its limited EPI activity, 1-BD is an attractive candidate as adjuvant to revitalize antibiotic therapy against resistant bacteria in a clinical setting. Structure–activity relationship studies will help to rationalize the design of 1-BD derivatives with enhanced and/or more specific activity.

Summary points

- 1-Benzyl-1,4-diazepane (1-BD) acts synergistically with levofloxacin and other antibiotics, enhancing their effects by two- to eightfold in *Escherichia coli* strains overexpressing efflux pumps AcrAB and AcrEF.
- 1-BD promotes the accumulation of ethidium bromide in the same *E. coli* strains.
- 1-BD increases membrane permeability, but only slightly affects membrane potential of *E. coli* cells.
- 1-BD halved the expression of *acrB*, as evaluated by quantitative photoactivated localization microscopy.
- The mechanism of action of 1-BD appears to be of a mixed type and overall different from that of major reference efflux pump inhibitors.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fmb-2019-0296

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