Novel piperazine arylideneimidazolones inhibit the AcrAB-TolC pump in *Escherichia coli* and simultaneously act as fluorescent membrane probes in a combined real-time influx and efflux assay.

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In this study we tested five compounds belonging to a novel series of piperazine aryldeneimidazolones for their ability to inhibit the AcrAB-TolC efflux pump. The biphenylmethylene derivative (BM-19) and the fluorenylmethylene derivative (BM-38) were found to possess the strongest efflux pump inhibitor (EPI) activity in the AcrAB-TolC overproducing *Escherichia* (*E.*) coli strain 3-AG100, whereas BM-9, BM-27, and BM-36 had no activity at concentrations of up to 50 µM in a Nile red efflux assay. MIC microdilution assays demonstrated that BM-19 (intrinsic MIC 200 µM) was able to reduce at ¼ MIC the MICs of levofloxacin, oxacillin, linezolid and clarithromycin by 8-fold. BM-38 (intrinsic MIC 100 µM) was only able to reduce at ¼ MIC the MICs of oxacillin and linezolid by 2-fold. Both compounds markedly reduced the MICs of rifampicin (BM-19 32-fold and BM-38 4-fold) suggestive of permeabilization of the outer membrane as additional mechanism of action. Nitrocefin hydrolysis assays demonstrated that both compounds were in fact weak permeabilizers of the outer membrane in addition to their EPI activity.

Moreover, it was found that BM-19, BM-27, BM-36 and BM-38 acted as near-infrared-emitting fluorescent membrane probes, which allowed for using them in a combined influx and efflux assay and thus to track the transport of an EPI across the outer membrane by an efflux pump in real time. The EPIs BM-38 and BM-19 displayed the most rapid influx of all compounds, whereas BM-27, which didn’t act as EPI, showed the slowest influx.
INTRODUCTION

The *E. coli* AcrAB-TolC efflux pump is the best characterized resistance-nodulation-cell division (RND) pump (1) and is capable of extruding a wide variety of structurally diverse compounds, encompassing many clinically administered antibiotics (e.g. beta-lactams, fluoroquinolones, tetracyclines) (2). It is constitutively expressed under physiological conditions and upon exposure to antibiotics mutations in local or global regulator genes can occur, hence leading to overexpression of this efflux pump and a multidrug-resistance (MDR) phenotype (3).

To combat MDR, efflux pump inhibitors (EPIs) are an attractive option and several EPIs that act against the AcrAB-TolC efflux pump have already been described in the literature (4-16), among which arylpiperazine and arylmorpholine derivatives constitute some of the largest systematically examined compound classes.

In this study we set out to test five compounds belonging to a novel series of piperazine derivatives of arylideneimidazolones for their ability to inhibit the AcrAB-TolC efflux pump. Moreover, since they displayed several structural features reminiscent of fluorescent charge-transfer-complexes, we opted to test all of them in a fluorescent spectral scan in whole cells to establish whether these compounds could be used in membrane transport assays.

MATERIALS AND METHODS

**Bacterial strains and culture media.** For the fluorescent and MIC assays described below, *E. coli* strains 3-AG100 (a multidrug-resistant mutant (*gyrA marR*) with *acrB* overexpression obtained from *E. coli* K-12 strain AG100 after repeated exposure to a fluoroquinolone) (3) and the *acrB* deletion strain 3-AG100ΔacrB (17) were used. The *Pseudomonas aeruginosa* PAO1 derivatives PA1426 (*oprD::ΩTc*, overproducing MexAB-OprM, selected after exposure to carbenicillin) and PA1425 (*oprD::ΩTc oprM::ΩHg*)(18) were a generous gift...
from Thilo Köhler (Geneva, Switzerland) and were used to test in MIC assays if the novel compounds could also potentiate the activity of antibiotics in non-fermenting bacteria. The *acrB* deletion strain KUN9180Δ*acrB* was generated from the extended-spectrum beta lactamase (ESBL) *E. coli* strain KUN9180 (a generous gift from Yasufumi Matsumura (Kyoto, Japan)) using the “Quick & Easy *E.coli* Gene Deletion Kit (Red®/ET® Recombination)” from Gene Bridges (Heidelberg, Germany) following the instructions by the manufacturer.

The strains were cultivated in either LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) for the fluorescent assays or in Müller-Hinton broth for the MIC microdilution assays. Details are given below.

**Synthesis of the piperazine arylideneimidazolones.** The piperazine arylideneimidazolones BM-9, BM-19, BM-27, BM-36 and BM-38 (Table 1) were synthesized according to the detailed information given in the supplementary data.

Briefly, the final compounds were obtained within 3- or 4-step synthesis route, including: (i) Knoevenagel condensation of 2-thiohydantoin with appropriate aromatic aldehydes, (ii) S-methylation process and (iii) condensation with suitable piperazines: 1-(2-hydroxyethyl)piperazine (BM-9, BM-27) or 1-acetyl-piperazine (acetyl protected forms of BM-19, BM-36 and BM-38). The deacetylation of the protected compounds BM-19, BM-36 and BM-38 was accomplished by acid hydrolysis. All five compounds were converted into hydrochlorides by saturation with gaseous HCl. In the syntheses, the following commercial chemical compounds were used: 2-thiohydantoin (Sigma-Aldrich, Poznań, Poland), biphenyl-4-carboxaldehyde (Sigma-Aldrich, Poznań, Poland), fluorene-2-carboxaldehyde (Sigma-Aldrich, Poznań, Poland), naphthalene-2-carboxaldehyde (Sigma-Aldrich, Poznań, Poland), methyl iodide (Sigma-Aldrich, Poznań, Poland), 1-(2-hydroxyethyl)piperazine (Sigma-
Aldrich, Poznan, Poland), 1-acetylpiperazine (Alfa-Aesar, Chemat, Gdańsk, Poland). Purity and identity of the synthesized compounds were evaluated by the use of $^1$H-NMR, IR spectroscopy, elemental analyses, TLC and melting point measurements.

Synthesis and characteristics of compound BM-9 and its intermediates were described earlier (19-23). Chemical procedures and characteristics of BM-19, BM-27, BM-36 and BM-38 are presented in the supplementary data.

**Other chemicals.** Carbonyl cyanide m-chlorophenylhydrazone (CCCP), phenyl-arginine-β-naphthylamide (PAβN) and Nile red were obtained from Sigma-Aldrich (Taufkirchen, Germany). Nitrocefin was ordered from Cayman Chemical (Ann Arbor, MI, USA).

**Susceptibility testing.** The MICs of a range of antimicrobial agents in the presence and absence of the various compounds were determined in a 96-well microtiter plate using strains incubated overnight at 37°C in a volume of 100 µL/well by a standard Müller-Hinton broth microdilution procedure and a final inoculum of 5 X 10$^5$ CFU/ml in accordance with CLSI/NCCLS M100-S20 guidelines (available at http://www.clsi.org). MIC testing was done in triplicate. Custom 96-well microtiter plates containing selected antimicrobials at increasing concentrations were purchased from Merlin Diagnostics (Bornheim, Germany).

**Nitrocefin hydrolysis assays**

To determine, whether BM-19 and BM-38 are capable of permeabilizing the outer membrane of *E. coli*, ESBL strain KUN9180ΔacrB was cultivated in LB broth, centrifuged 8 min at RT, 4000 rpm, washed twice in phosphate buffered saline (PBS) and then resuspended in PBS containing 0.4% glucose with or without 1mM MgCl$_2$ until an OD$_{600nm}$ of 0.5 was reached.
Thereafter, nitrocefin (final concentration 32 µg/ml) was added to the bacterial suspension in the absence or presence of the EPIs BM-19, BM-38 or PAβN (final concentration 50 µM) and nitrocefin hydrolysis was monitored spectrophotometrically (increase in absorbance at 490 nm) using an Infinite 200Pro (Tecan, Crailsheim/Germany) 96-well plate reader. Nile red efflux assay in the absence and presence of the piperazine arylideneimidazolone EPIs. The protocol for the Nile red efflux assay has been published previously (24) and all procedures were carried out, accordingly. Briefly, the cells were cultivated overnight in LB broth to de-energize them. Then, after a washing step they were resuspended in the potassium phosphate buffer and the desired piperazine arylideneimidazolone was added 15 min after the addition of 5 µM CCCP at a standard concentration of 50 µM to screen for activity in the preliminary experiments. Nile red efflux was initiated by addition of glucose. In case an effect on Nile red efflux was observed, these compounds were re-tested at different concentration ranges from threshold activity to complete abolishment of dye efflux. All assays were at least carried out in triplicate. Since the piperazine arylideneimidazolone efflux experiments demonstrated that these compounds were properly retained within the cell envelope in the de-energized state, we did not add them to the cell suspension after the washing step. In the upper concentration range quenching phenomena of varying degrees were observed and compensated for by adjusting the pre-energization fluorescence intensity to 100 relative fluorescence units. Prior to the experiments, it was established that no compound displayed any considerable autofluorescence with the bacterial cells using the Nile red excitation and emission wavelengths.

Preparation of bacterial cells for the fluorescent assays with the piperazine arylideneimidazolones
A single bead of the 3-AG100 -80°C frozen stock (maintained in Cryobank vials from Mast Diagnostica GmbH, Reinfeld, Germany) was directly added to 20 mL of LB broth in an Erlenmeyer flask and grown on a shaker (200 rpm; 37°C) for 16-18 hours. Thereafter, a 10 mL portion of the culture was centrifuged at 4,000 rpm for 5 min at room temperature. The pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM MgCl₂ (PPB). After another centrifugation and resuspension step, the cells were adjusted to an OD₆₀₀ nm of 0.25 in PPB.

Fluorescent spectral scans of the piperazine arylideneimidazolones in the presence of strain 3-AG100.

The 3-AG100 cell suspension was prepared as described above and 2 mL were transferred to a glass cuvette in a Perkin Elmer (Waltham, MA, USA) LS55 spectrofluorimeter. CCCP (final concentration 10 µM) and the respective piperazine arylideneimidazolone (final concentration 10 µM) were added and allowed to rest for 15 min at room temperature. The mixture was stirred with a magnetic stirrer.

A fluorescent spectral scan was performed using an excitation wave length from 200 through 420 nm based on 10 nm steps. The emission spectrum was recorded from 200 through 900 nm.

Thereafter, the spectral scan was repeated in the energized state after adding glucose (final concentration 50 mM) to the cell suspension.

BM-19, BM-27, BM-36 and BM-38 combined real-time influx and efflux assay
The cell suspension was prepared as described above and 2 mL were transferred to a glass cuvette in a Perkin Elmer LS55 spectrofluorimeter. The mixture was stirred with a magnetic stirrer.

The fluorescence intensity was recorded over a time-course of 2000 s using the wavelength settings given in Table 1. CCCP was added to a final concentration of 5 µM at 50 s. BM-19, BM-27, BM-36 or BM-38 were then added to a final concentration of 5 µM at 500 s. At 1500 s efflux of the respective dye was triggered by the addition of glucose (final concentration 50 mM). All assays were at least carried out in triplicate.

**BM-19, BM-27, BM-36 and BM-38 modified real-time efflux assay**

Alternatively, the piperazine arylideneimidazolone real-time efflux assay was performed as described previously for the fluorescent membrane probe 1,2’-DNA (25). Briefly, after loading the de-energized cells with the dye, the cells were centrifuged and washed once in PPB to remove CCCP and the dye from the extracellular PPB. Thereafter, the fluorescence intensity was recorded and glucose was added for energization as described above.

**Cytotoxicity assays**

Human liver carcinoma HepG2 cell viability after 72h of incubation with the piperazine arylideneimidazolones and the reference cytostatic drug doxorubicine was determined by performing the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega GmbH, Mannheim, Germany).

Cells were seeded on a 96-well plate at a concentration of 75 cells/µl in a total volume of 200 µl of medium, 24h prior to the experiment. On the next day, the cell culture medium was...
removed and fresh medium was placed in the microplate wells. The piperazine aryldeneimidazolones were then added at a final concentration of 0.03 to 100 μM for BM-9 and 0.3 to 100 μM for the other compounds, whereas doxorubicin was tested in a concentration range from 0.01 to 10 μM. DMSO concentration in the assay was 1%.

After the incubation period, the medium containing the piperazine aryldeneimidazolones or doxorubicin was removed and 120 μl of fresh medium containing MTS dye/phenazine methosulfate solution was added to each well. After 4 h of further incubation, absorbance was measured at 490 nm directly from 96-well assay plates. Wells containing no cells were used as blank samples, whereas wells containing 1% DMSO were taken as non-treated controls of fully viable cells.
RESULTS

Antimicrobial activity of the piperazine arylideneimidazolones in the E. coli and P. aeruginosa strains overproducing or lacking a major RND efflux pump.

The MICs of the various compounds for the E. coli and P. aeruginosa strains are given in Table 2. BM-38 was found to be the compound with the lowest MIC in the AcrAB-TolC overproducing E. coli strain 3-AG100 (100 µM), whereas BM-9 displayed MICs greater than 1600 µM. Both P. aeruginosa strains displayed the same MICs (400 µM) for BM-19 and BM-38.

BM-19 and BM-38 are weak permeabilizers of the outer membrane in an acrB-deficient E. coli strain

BM-19 and BM-38 (50 µM) were found to weakly permeabilize the outer membrane of E. coli ESBL strain KUN9180ΔacrB as determined by a nitrocefin hydrolysis assay in the absence of MgCl₂ in the cell suspension (Fig 1a). BM-19’s effect was slightly more pronounced than that of BM-38. PAβN however, was found to markedly enhance nitrocefin hydrolysis in MgCl₂ free cell suspension, as described previously (13). When the experiments were carried out in medium containing 1mM MgCl₂ the nitrocefin hydrolysis curves of all three EPIs approached the no-EPI control curve (Fig. 1b).

BM-19 and BM-38 inhibit Nile red efflux in the acrAB overexpressing strain 3-AG100.

BM-9, BM-27, and BM-36 had no impact on Nile red efflux at concentrations of up to 50 µM. However, it was found that both BM-19 and BM-38 inhibited Nile red efflux in a dose-dependent manner (Fig. 2a+b). The first effects were seen at 25 µM and at 100 µM efflux was completely abolished.
BM-19 and BM-38 potentiate the activity of various antibiotics in *E. coli* but have very poor activity in *P. aeruginosa*.

Since BM-9, BM-27 and BM-36 had no impact on *E. coli* Nile red efflux at concentrations up to 50 µM (see above), we opted to examine only BM-19 and BM-38 for their ability to sensitize our 3-AG100 *E. coli* strain to various antibiotics. All results are given in Table 3.

BM-38 (MIC in 3-AG100 100 µM), as described above, had been found to possess the best antimicrobial activity but did only display relatively weak potentiating activity. At ¼ MIC, BM-38 reduced the MIC of rifampicin by 4-fold, and the MICs of oxacillin and linezolid by two-fold, but it had no effect on the MICs of the other tested antibiotics even when the concentration of BM-38 was raised to ½ MIC.

However, BM-19 which possessed less antimicrobial activity (MIC in 3-AG100 200 µM) was found at ¼ MIC to potentiate the antimicrobial activity of all of the tested antibiotics – it reduced the MIC of rifampicin by 32-fold, the MICs of levofoxacin, chloramphenicol, oxacillin, linezolid and clarithromycin by 8-fold, and the MIC of tetracycline by 4-fold.

The *acrB* deletion strain 3-AG100 Δ*acrB* demonstrated dose-dependent MIC reductions with both piperazine arylideneimidazolones and most tested antibiotics except for levofoxacin and chloramphenicol.

Finally, we carried out potentiating assays with our two *P. aeruginosa* strains, but found very poor activity (two-fold MIC reductions for rifampicin in PA1426 and two-fold MIC reductions for linezolid and clarithromycin in PA1425).
The piperazine arylideneimidazolones act as fluorescent membrane probes and are extruded by the AcrAB-TolC efflux pump of 3-AG100.

With the exception of BM-9 (which also did only display a weak fluorescence signal upon dye loading) every compound produced different spectra in the de-energized versus the energized state using whole cells and possessed a second emission maximum in the near-infrared range. The BM-27 spectral scans are depicted in Fig. 3. The optimal excitation and emission wavelengths for the individual dyes are given in Table 1.

Using this data, combined influx and efflux assays could be performed. Optimization experiments determined that a CCCP concentration of 5-10 µM and a dye concentration of 5 - 10 µM gave the highest ratio between fluorescence intensity in the dye-loaded de-energized state and in the energized state (data not shown). Of all compounds tested, BM-27 was found to be the dye which showed the most dramatic drop in fluorescence intensity after energization of the cells (Fig. 4). The other compounds demonstrated a more moderate but still clearly visible drop in fluorescence intensity after onset of efflux.

The influx rates differed considerably between the dyes in the following order from fast influx to slow influx: BM-38 > BM-19 > BM-36 > BM-27.

Using CCCP concentrations of around 5 µM for the combined influx and efflux assays, efflux could easily be triggered by the addition of 50 mM glucose. Performing a more classical efflux assay, where most of the CCCP was removed by a washing step before energization, it could be demonstrated that the dyes were easily retained in the cell envelope like demonstrated previously with the dyes Nile red (24) and 1,2'-DNA (25). The washing step led to a moderate acceleration of dye efflux (Fig. 5) compared with the combined influx and efflux assay, where small amounts of CCCP were still present in the buffer. In the ΔacrB...
strain dye efflux was almost completely abolished. **Cytotoxicity of the piperazine aryldeneimidazolones**

The evaluated compounds showed moderate to weak anti-proliferative activity towards human liver carcinoma cell line HepG2 (values given in Fig. 6). The IC$_{50}$ values determined for the compounds were between 25-fold and 90-fold higher than the IC$_{50}$ for the reference cytotoxic drug doxorubicin. Nevertheless, cytotoxicity was demonstrated for BM-19 and BM-38 at concentrations used for efflux pump inhibition (25 to 50 µM).

One of the tested compounds, BM-9, dose-dependently stimulated the growth of the evaluated cell line.

**DISCUSSION**

The novel piperazine aryldeneimidazolones BM-19 and BM-38 were found to act as EPIs in a Nile red efflux assay using an AcrAB-TolC overproducing *E. coli* strain. In line with these findings, potentiating activity was observed with certain antibiotics in an MIC microdilution assay.

However, BM-38’s activity as an EPI was overshadowed by its relatively potent intrinsic antimicrobial activity (MIC 100 µM in contrast to 200 µM in BM-19). When comparing both compounds at their respective ¼ intrinsic MIC, BM-19 (which displayed less antimicrobial activity than BM-38) showed all the characteristics of a broad-spectrum EPI, namely it reduced the MICs of almost all tested antibiotics by 4- to 8-fold, coming close to, but not reaching the MICs of the ∆acrB strain. Rifampicin was the only exception, since BM-19 reduced its MIC by 32-fold and thus below the MIC of the ∆acrB strain, suggesting that this compound might possess additional mechanisms of action (e.g. permeabilization of the outer membrane). A similar, but somewhat weaker effect was found with BM-38 and rifampicin. To determine if the potentiation of rifampicin activity could be due to
permeabilization of the outer membrane (an effect already described for the model EPI PAβN (13)) we carried out standard nitrocefin hydrolysis assays and indeed showed that both BM-19 and BM-38 are permeabilizers in MgCl₂ free medium, albeit much weaker than PAβN, and that the outer membrane can withstand this action by addition of 1 mM MgCl₂.

Besides the good potentiation of rifampicin activity, BM-38 at ¼ intrinsic MIC affected only the MICs of oxacillin and linezolid by two-fold in 3-AG100. Even raising the concentration to ½ intrinsic MIC (and thus to the same maximum concentration used in the BM-19 assay) could only moderately increase the antimicrobial activity of oxacillin and linezolid and failed to demonstrate any activity with levofoxacin and clarithromycin. Like the model piperazine EPI NMP, our novel piperazine arylideneimidazolones have very poor EPI activity in <i>P. aeruginosa</i>—which is probably due to the different outer membrane structure of this species with greatly reduced influx rates.

Considering structural properties of the five arylideneimidazolones investigated, BM-19, BM-36 and BM-38 represent the amphiphilic group with unsubstituted positive ionisable piperazine nitrogen at position 2 of imidazolone, placed opposite to the bulky double-aromatic-methylidene system at position 5. Both, BM-19 and BM-38 display a more extended aromatic area than the closely fused naphthalene rings BM-36. However, a difference in flexibility of the 5-aromatic moieties of both the active compounds is seen. In the case of BM-19, the single bond between both phenyl rings of the biphenyl gives some rotary freedom, which might facilitate a good fit within the AcrB binding pocket. The fluorene aromatic moiety of BM-38 includes an additional methylene bridge between the two phenyl rings, which blocks the freedom of mutual rotation of the rings in comparison to that of BM-19. Thus, the 5-aromatic area of the compound BM-38 creates a spatial hindrance of the fused triple rings that might significantly impact the fit within the AcrB binding pocket. We therefore hypothesize that the more rigid structure of BM-38 may make it harder for this...
compound to interact with different target sites within the AcrB binding pocket and might thus affect the transport of fewer substrates.

Although BM-19 appears to be a broad-spectrum EPI, it is definitely not an “ideal EPI” like 1-(1-naphthylmethyl)-piperazine (NMP) (6), which would only reduce the substrate MICs in an AcrAB-ToIc overproducing strain and not in the respective efflux-deficient strain. In contrast, both BM-19 and BM-38 were found to markedly potentiate the activity of the tested antibiotics in the ΔacrB strain, suggesting additional mechanisms like the – albeit moderate – permeabilization of the outer membrane.

Moreover, we serendipitously discovered that most of the piperazine aryldieneimidazolones are also environment-sensitive fluorescent membrane probes as well as substrates of the AcrAB-ToIc efflux pump. Some of them, especially BM-27, can thus be excellently used for real-time efflux assays, where cells are loaded with a dye in the de-energized state and efflux is triggered upon energization of the cells. Like 1,2’-DNA (25), the piperazine aryldieneimidazolones are capable of emission in the near-infrared range of the electromagnetic spectrum, where the signal-to-noise ratio in biological samples is typically high due to low autofluorescence. Contrary to the highly hydrophobic dyes 1,2’-DNA and Nile red however, this new group of dyes is readily water-soluble, so that no organic solvents have to be used, which may adversely affect dye efflux. Moreover, the hydrophobicity may pose problems with adhesion to reaction vessels thus rendering the use in a reproducible influx assay almost impossible. Due to their amphiphilic nature, the piperazine aryldieneimidazolones are membrane probes for which combined influx and efflux assays can be readily performed.
Interestingly, compounds like BM-19 and BM-38 can play multiple roles to assess and modify transport of substrates across the outer membrane. First, they can be used as an EPI to block the transport of other substrates (as described above using the Nile red efflux assay). Second, their own transport across the outer membrane by an RND efflux pump can be monitored in real time due to their fluorescent membrane probe characteristics. Third, these compounds exert intrinsic antimicrobial activity and allow thus to relate their MIC to influx and efflux characteristics in a given strain. The background is that researchers typically compare MIC ratios established in RND pump overproducers versus the RND pump deficient strain to assess whether a given substrate is a good or poor substrate of an RND efflux pump. However, without knowledge of influx rates, MIC ratios tell us nothing about the corresponding efflux rates. In fact, a compound may be an excellent substrate of an RND efflux pump, but due to its high influx rate the pump may simply be overwhelmed by substrate accumulation leading to no or very little difference in MICs as demonstrated previously in a study on beta-lactam efflux (26).

From the MIC ratio between the AcrAB-TolC overproducing 3-AG100 strain (MIC 100 µM) and the ΔacrB strain (MIC 50 µM) - which is only 2 - one would conclude that BM-38 is a poor substrate of this pump. However, the real-time efflux assay clearly demonstrates that this compound is in fact an excellent substrate. As explained above, the influx of BM-38 seems to be quite fast (although this compound seemed to be a weaker permeabilizer than BM-19 in the nitrocefin assays) relative to the efflux rates and thus even a highly active efflux pump may not be capable of pumping out enough substrate to markedly decrease its MIC. In fact, since the piperazine arylideneimidazolone efflux assays can be easily combined with an influx assay, it was found that influx in de-energized cells is occurring quite rapidly with BM-38.
(more than 90 % dye accumulation reached about 100 s into an experiment), whereas it takes much longer (up to 1000 s) for BM-27, which is not an EPI. Hence, overwhelming the efflux pump by rapid influx of a substrate may be a good strategy to outcompete at least a subset of xenobiotics that use similar routes and recognition sites across the pump protein.

Unfortunately, as described previously (24, 25), the concentration-signal intensity-ratio of substrates used in fluorescence-based real-time efflux assays is not linear, so Michaelis-Menten kinetics cannot be easily derived to establish whether EPIs inhibit a pump protein in a competitive manner or not. However, it might be possible to determine what causes the non-linearity observed (e.g. self-quenching) and to compensate for this phenomenon using a numerical model.

To conclude: although BM-19 and BM-38 are cytotoxic to eukaryotic cells at the concentrations used for EPI activity (Fig. 6), they are interesting lead structures for synthesis of less cytotoxic compounds in the future. Moreover, the unique combination of fluorescent, EPI and antimicrobial properties makes them interesting basic researchs tools for elucidating molecular mechanisms of efflux pump inhibition - e.g. by random or site-directed mutagenesis of substrate binding pockets and subsequent fluorescent substrate / EPI transport assays.
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inhibited by chlorpromazine. Antimicrobial agents and chemotherapy 52:3604-3611.


Table 1. Basic properties of the piperazine arylideneimidazolones

<table>
<thead>
<tr>
<th>Cpd</th>
<th>Structure</th>
<th>Mol. Weight</th>
<th>Excitation Maximum (nm)</th>
<th>1st Emission Maximum (nm)</th>
<th>2nd Emission Maximum (nm)</th>
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<tbody>
<tr>
<td>BM-9</td>
<td>HCl</td>
<td>386.88</td>
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<td>b</td>
<td>b</td>
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<td>390</td>
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<tr>
<td>BM-27</td>
<td>HCl</td>
<td>424.92</td>
<td>400</td>
<td>457</td>
<td>873</td>
</tr>
<tr>
<td>BM-36</td>
<td>HCl</td>
<td>342.82</td>
<td>400</td>
<td>452</td>
<td>855</td>
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<tr>
<td>BM-38</td>
<td>HCl</td>
<td>380.87</td>
<td>400</td>
<td>457</td>
<td>873</td>
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</tbody>
</table>
measured in E. coli 3-AG100 cell suspension (OD$_{600nm}$ was 0.25)

b no excitation and emission maxima given since fluorescence intensity upon 3-AG100 dye-loading was found to be very low and no marked difference between the de-energized and energized state could be detected
Table 2. MICs of the piperazine arylidencimidazolones

<table>
<thead>
<tr>
<th>Strain</th>
<th>BM-9</th>
<th>BM-19</th>
<th>BM-27</th>
<th>BM-36</th>
<th>BM-38</th>
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<tr>
<td>3-AG100</td>
<td>&gt; 1600</td>
<td>200</td>
<td>&gt; 1600</td>
<td>800</td>
<td>100</td>
</tr>
<tr>
<td>3-AG100Δacr</td>
<td>&gt; 1600</td>
<td>100</td>
<td>&gt; 1600</td>
<td>400</td>
<td>50</td>
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<tr>
<td>PA1425</td>
<td>n.d.</td>
<td>400</td>
<td>n.d.</td>
<td>n.d.</td>
<td>400</td>
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<tr>
<td>PA1426</td>
<td>n.d.</td>
<td>400</td>
<td>n.d.</td>
<td>n.d.</td>
<td>400</td>
</tr>
</tbody>
</table>

*not done
**TABLE 3. Effect of the EPIs BM-19 and BM-38 on various antibiotic MICs (mg/L)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/L)</th>
<th>Levofloxacin</th>
<th>Tetracycline</th>
<th>Chloramphenicol</th>
<th>Rifampicin</th>
<th>Oxacillin</th>
<th>Linezolid</th>
<th>Clarithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-AG100 without EPI</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>512</td>
<td>512</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>3-AG100 + BM-19 25 µM</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>512</td>
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<td>3-AG100 + BM-19 50 µM (¼ MIC)</td>
<td><strong>0.13</strong></td>
<td><strong>1</strong></td>
<td><strong>2</strong></td>
<td><strong>0.5</strong></td>
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<td><strong>64</strong></td>
<td><strong>16</strong></td>
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<tr>
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<td>4</td>
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<td>4</td>
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<td>256</td>
<td>128</td>
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<tr>
<td>3-AG100 + BM-38 50 µM</td>
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<td><strong>2</strong></td>
<td><strong>8</strong></td>
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<tr>
<td>3-AG100ΔacrB without EPI</td>
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<td>&lt;=0.06</td>
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<td>128</td>
<td>16</td>
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<td>256</td>
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<td>16</td>
<td>2048</td>
<td>2048</td>
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<td>16</td>
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<td>16</td>
<td>64</td>
<td>32</td>
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* MICs which differ in the presence of BM-19 or BM-38 by a least 4-fold from the AcrAB-TolC overproducing strain 3-AG100 in the absence of these compounds are given in boldface.
FIGURE LEGENDS

Fig 1a: Impact of PAβN, BM-19 and BM-38 on outer membrane permeabilization of *E.coli* KUN9180ΔacrB as measured by nitrocefin hydrolysis (OD₄₉₀nm) in the absence of MgCl₂.

Fig 1b: Impact of PAβN, BM-19 and BM-38 on outer membrane permeabilization of *E.coli* KUN9180ΔacrB as measured by nitrocefin hydrolysis (OD₄₉₀nm) in the presence of 1mM MgCl₂.

Fig. 2a. Dose-dependent inhibition of real-time Nile red efflux by BM-38 in the *acrAB*-overexpressing strain 3-AG100. Energization with 50 mM glucose at t = 50 s. Pre-energization fluorescence intensity adjusted to 100 relative fluorescence units.

Fig. 2b. Dose-dependent inhibition of real-time Nile red efflux by BM-19 in the *acrAB* overexpressing strain 3-AG100. Energization with 50 mM glucose at t = 50 s. Pre-energization fluorescence intensity adjusted to 100 relative fluorescence units.

Fig. 3. Emission spectra of BM-27 (10 µM) in a bacterial suspension of 3-AG100 (OD₆₀₀ nm 0.25) in the de-energized and energized state. Excitation wavelength was 400 nm.

Fig. 4. Combined real-time influx and efflux assays using the different piperazine arylideneimidazolones as fluorescent membrane probes in the *acrAB*-overexpressing strain 3-AG100. 5 µM CCCP was added to de-energized cells at t = 50 s, 5 µM dye was added at t = 500 s and efflux was triggered by addition of 50 mM glucose at t = 1500 s. Identical conditions for all dyes (OD₆₀₀nm 0.25, near infrared emission maximum as given in Table 1, slit width 10 nm).

Fig. 5. Real-time efflux assays using the different piperazine arylideneimidazolones as fluorescent membrane probes in the *acrAB*-overexpressing strain 3-AG100. After dye loading for 30 min the de-energized cells were washed once in PPB and efflux was triggered.
by addition of 50 mM glucose at $t=50$ s. Identical conditions for all dyes ($OD_{600nm}$ 0.25, near infrared emission maximum as given in Table 1, slit width 10 nm). The 3-AG100$\Delta acrB$ strain demonstrates that BM-27 efflux in the 3-AG100 strain is mainly mediated by the AcrAB-TolC pump.

Fig. 6. HepG2 cells viability after 72h of incubation with the above compounds using the CellTiter 96 AQueous non-radioactive cell proliferation assay.