in vivo *26*: 223-230 (2012)

The Activity of 16 New Hydantoin Compounds on the Intrinsic and Overexpressed Efflux Pump System of *Staphylococcus aureus*

ANNA DYMEK[1], ANA ARMADA[2], JADWIGA HANDZLIK[1], MIGUEL VIVEIROS[2,3], GABRIELLA SPENGLER[4], JOSEPH MOLNAR[3,4], KATARZYNA KIEĆ-KONONOWICZ[1,3] and LEONARD AMARAL[2,3]

[1]*Department of Technology and Biotechnology of Drugs, Jagiellonian University, Medical College, Krakow, Poland;*
[2]*Group of Mycobacteriology, Unit of Medical Microbiology, Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal;*
[3]*COST Action BM0701 (ATENS) of the European Commission, Brussels, Belgium;*
[4]*Institute of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary*

Abstract. *Aim: To evaluate a new series of 16 hydantoin derivatives for activity against the intrinsic and over-expressed efflux pumps of the ATTC 25923 Staphylococcus aureus and the clinical Staphylococcus aureus HPV-107 strain, respectively. Materials and Methods: The hydantoin compounds were evaluated for activity against the efflux pumps of the ATTC 25923 S. aureus and the clinical S. aureus HPV-107 strains by the aid of the automated ethidium bromide method. Compounds that inhibited the efflux pumps of either strain were evaluated for ability to reduce or reverse resistance of these strains to oxacillin. Results: Although most of the hydantoins inhibited the efflux pumps of the ATTC strain, none reduced the resistance of this strain to oxacillin. In contrast, the inhibition of the Qac efflux pump present in HPV-107 was inhibited to some degree, by much higher concentrations of the hydantoin compounds than that needed for similar activity against the ATTC strain; only hydantoin PI8a significantly reduced the minimum inhibitory concentration of oxacillin against the HPV-107 strain. Conclusion: Hydantoin compound PI8a may have potential for therapy of a methicillin-resistant S. aureus infection whose multidrug-resistant phenotype is due to overexpression of an efflux pump.*

Correspondence to: Leonard Amaral, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal. Tel: +351 213652600, Fax: +351 213632105, e-mail: lamaral@ihmt.unl.pt

Key Words: Staphylococcus aureus ATCC 25923 wild-type, *Staphylococcus aureus* HPV-107, intrinsic efflux pump, Qac efflux pump, hydantoin derivatives, oxacillin, inhibition of efflux pumps, reversal of resistance to oxacillin.

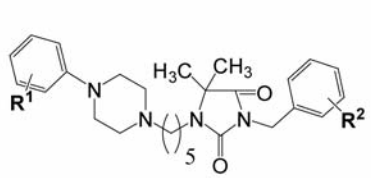
Hydantoins play an important role in the purine catabolic pathway that regulates the purine pool in the cell to provide precursors for nucleic acid synthesis (1), have essential metabolic function because they hydrolyse hydantoin and 5'-monosubstituted hydantoin derivatives, are valuable in the production of optically pure amino acids (2) and are essential components of salvage pathways for nucleobases and related metabolites, *e.g.* nucleobase-cation-symport-1 (NCS1) benzylhydantoin transporter, Mhp1 from *Microbacterium liquefaciens* (3). Our recent studies show that hydantoins have the ability to inhibit the efflux pump of cancer cells (4), as well as the efflux pump system of *Salmonella enterica* serovar enteritidis (5).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen (6) that makes therapy problematic when it colonises cardiac valves (6). Most clinical isolates of MRSA are known to have a multidrug-resistant phenotype (7) that is due to an overexpression of an efflux pump (8, 9). Consequently, we have evaluated the effects of 16 new hydantoin compounds on the intrinsic efflux pump system of wild-type *Staphylococcus aureus* and an MRSA strain that overexpresses its efflux pump.

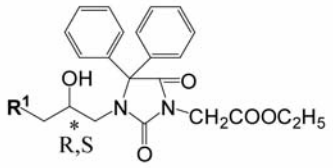
Materials and Methods

Compounds and materials. Sixteen hydantoin derivatives were synthesized by methods to be presented elsewhere and were evaluated for inhibitory effects on the efflux pump systems of *S. aureus*. The hydantoin compounds and their structures are listed in Table I. Each contained two aromatic rings and were divided into four groups (A-C and MF8) according to the position of each aromatic fragment. Group A comprised 11 phenylpiperazine 5,5-dimethylhydantoin derivatives (PI1a-PI11a) possessing two terminal (un)substituted phenyl rings within substituents at N1 and

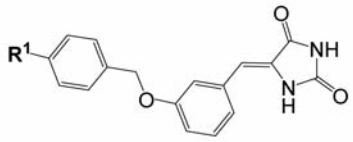
Table I. *Structure of hydantoin derivatives tested (PI1a-11a, KF4, KF20 HY83, HY84 and MF8).*



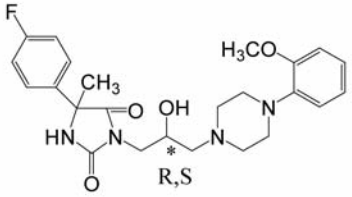
A



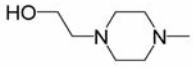
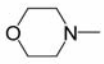
B



C



MF-8

Compound	Group	R^1	R^2	Compound	Group	R^1	R^2
PI1a	A	H	H	PI9a	A	2,3-diCl	2,4-diCl
PI2a	A	2-OCH_3	H	PI10a	A	3,4-diCl	2,4-diCl
PI3a	A	3-OCH_3	H	PI11a	A	4-Cl	2,4-diCl
PI4a	A	2-F	H	KF4	B		-
PI5a	A	4-F	H	KF20	B		-
PI6a	A	2,4-diF	H	HY83	C	Cl	-
PI7a	A	2,4-diF	4-F	HY84	C	H	-
PI8a	A	4-F	4-F				

N3 positions of the hydantoin. Group B included two racemic N3-ester derivatives of 5,5-diphenylhydantoin with 2-hydroxypropyl linker between hydantoin and hydroxyethylpiperazine (KF4) or morpholine (KF20). Two 5-arylidene derivatives (HY83 and HY84), possessing two benzene moieties connected to each other by ether linker, belonged to group C. Aromatic rings of compound MF8 are found at position 5 and at the terminal part of the amineralkyl substituent at position N3 of the hydantoin structure. Syntheses of compounds KF4, KF20, HY83 and HY84 have been described elsewhere (10, 11). Syntheses of compounds PI1a-PI11a and MF8 involved two different pathways and will be described elsewhere. Purity and identity of the 15 hydantoin derivatives were confirmed by the use of spectroscopic methods [nuclear magnetic resonance spectroscopy (^{1}H-NMR), infrared spectroscopy (IR), elemental analysis, melting point measurement and thin-layer chromatography (TLC)].

The compounds were dissolved in dimethyl sulfoxide (DMSO). Ethidium bromide (EB) was purchased from Sigma (Madrid, Spain). Tryptic soy in powder form was purchased from Difco (Madrid, Spain) for the preparation of broth and agar.

Bacteria. Wild-type *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 and the HPV-107 MRSA strain (representative of the MRSA Iberian clone, was isolated at a Portuguese hospital in 1992 and is characterised by resistance to

several classes of antibiotics, particularly β-lactams, aminoglycosides, fluoroquinolones, macrolides, rifampicin and tetracycline) whose partial resistance to oxacillin is due the presence of a plasmid that contains the gene coding for the Qac efflux pump (9). The HPV-107 strain was graciously provided by Professor Dr. H. De Lencastre.

Determination of minimum inhibitory concentrations (MICs). The MICs of compounds, including EB, were determined by using broth microdilution method in Mueller-Hinton Broth (MHB), according to Clinical and Laboratory Standards Institute (CLSI) recommendations (12). The MICs were required in order that the concentrations of each hydantoin used in the study would be no greater than ½ or ¼ of its MIC, and hence, would not affect the viability of the bacteria (13, 14).

Real-time EB accumulation assay. The activity of compounds on the real-time accumulation of EB was assessed by the automated EB method, previously described in detail elsewhere (13, 14), using a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Australia). Briefly, strains were cultured in tryptic soy broth (TSB) medium until they reached an optical density (OD) of 0.6 at 600 nm; they were then centrifuged at 10000× *g* for 3 minutes, the pellets were re-suspended in phosphate-buffered saline (PBS) (pH 7.4) with a final concentration of glucose of 0.4% and the OD adjusted to 0.6 at 600 nm. Aliquots of 45 μl of the cell suspension were distributed to 0.2 ml tubes. The compounds were individually added at different concentrations (50 and 200 mg/l final concentration) in 5 μl volumes of their stock solutions, and finally 45 μl of EB was added to yield a final concentration of 1 mg/l (Sigma-Aldrich Quimica SA, Madrid, Spain) in PBS with glucose. It is important to note that prior to the experiments described, the maximum concentration of EB which was within the capacity of the bacterium to extrude was determined and shown to be exactly that previously reported (13, 14). For the wild-type reference bacterial strain employed in the study, this concentration of EB was determined to be 0.25 mg/l (9). The tubes were placed into a Rotor-Gene 3000™ thermocycler and the fluorescence monitored on a real-time basis.

From the real-time data, the activity of the compound, namely the relative final fluorescence (RFF) of the last time point (minute 20) of the EB accumulation assay was calculated according to the formula:

$$relative\ fluorescence\ index\ (RFI) = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

where $RF_{treated}$ is the relative fluorescence (RF) at the last time point of the EB retention curve in the presence of an inhibitor; $RF_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). The greater the difference between $RF_{treated}$ and $RF_{untreated}$, the greater the degree of the EB accumulated and, therefore, the greater the degree of inhibition of the efflux pump system of the bacterium by the hydantoin compound. Dividing the RFI by the number of micromoles of the hydantoin compound used in the assay provides a specific activity of inhibition of the efflux pump of the bacterium. Because the overexpressed efflux pump system is due to an increase of efflux pumps, the amount of hydantoin needed to inhibit the efflux pump system is greater than that needed to inhibit the intrinsic efflux pump of the wild-type strain.

Determination of the reduction of resistance to oxacillin by compounds that inhibit the efflux pump of the ATCC and HPV-107 S. aureus strains. The MIC of oxacillin was first determined with the aid of the microplate broth dilution method (12). The MIC for oxacillin was then assessed in the absence and presence of concentrations of the compounds that significantly inhibited the efflux pump (*i.e.* produced accumulation of EB). Reduction of resistance to oxacillin was considered to be significant when the MIC of oxacillin was reduced by at least four-fold.

Results

The ATCC wild-type strain has an intrinsic efflux pump system and the HPV-107 strain has a plasmid containing the Qac gene which renders the bacterium multidrug-resistant (9). The result of the determination of MICs of the hydantoin derivatives indicated that the compounds did not affect the viability of the strains at concentrations as high as 200 mg/l. Higher concentrations produced differing degrees of precipitation and were therefore not used in this study. As previously mentioned, the MIC of each hydantoin identifies the concentration that does not affect the viability of the bacterium (13). Hence concentrations at or below the 200 mg/l level were selected for the study of their effects on the efflux pump system of the strains.

The concentration of EB that is barely extruded by the ATTC wild-type and the HPV-107 strains is shown by Figures 1A and B. Note that whereas the highest concentration of EB that does not result in increasing accumulation during the 20 minutes of the assay by the wild-type ATCC strain is 0.25 mg/l (Figure 1A), accumulation of EB by the HPV-107 strain does not take place even at the very high concentration of 2 mg/l (Figure 1B). Note that the degree of fluorescence for each of the concentrations is due to that produced by the concentration of EB itself. These results are consistent with the expectation that less accumulation of an efflux pump substrate will take place when an efflux pump system is overexpressed or present beyond that of the intrinsic system (7-9, 13, 14). These results afforded the selection of a concentration of EB in the assay that would barely be accumulated by each of the *Staphylococcus* strains. This represents the highest concentration of EB that the cell can extrude before it begins to accumulate EB. Concentrations of 0.25 and 2.0 mg/l of EB were selected for the assay of the effects of each hydantoin compound on the efflux pump system of the ATTC and HPV-107 strains, respectively.

The evaluations of hydantoin derivatives for activity against the intrinsic efflux pump of the wild-type ATTC and the Qac efflux pump of the HPV-107 strain are represented by the examples provided by Figure 2A and B. Briefly, hydantoin derivative PI7a promotes the accumulation of EB by the wild-type ATCC strain in a concentration-dependent manner (Figure 2A). As showed in Figure 2B, although this same

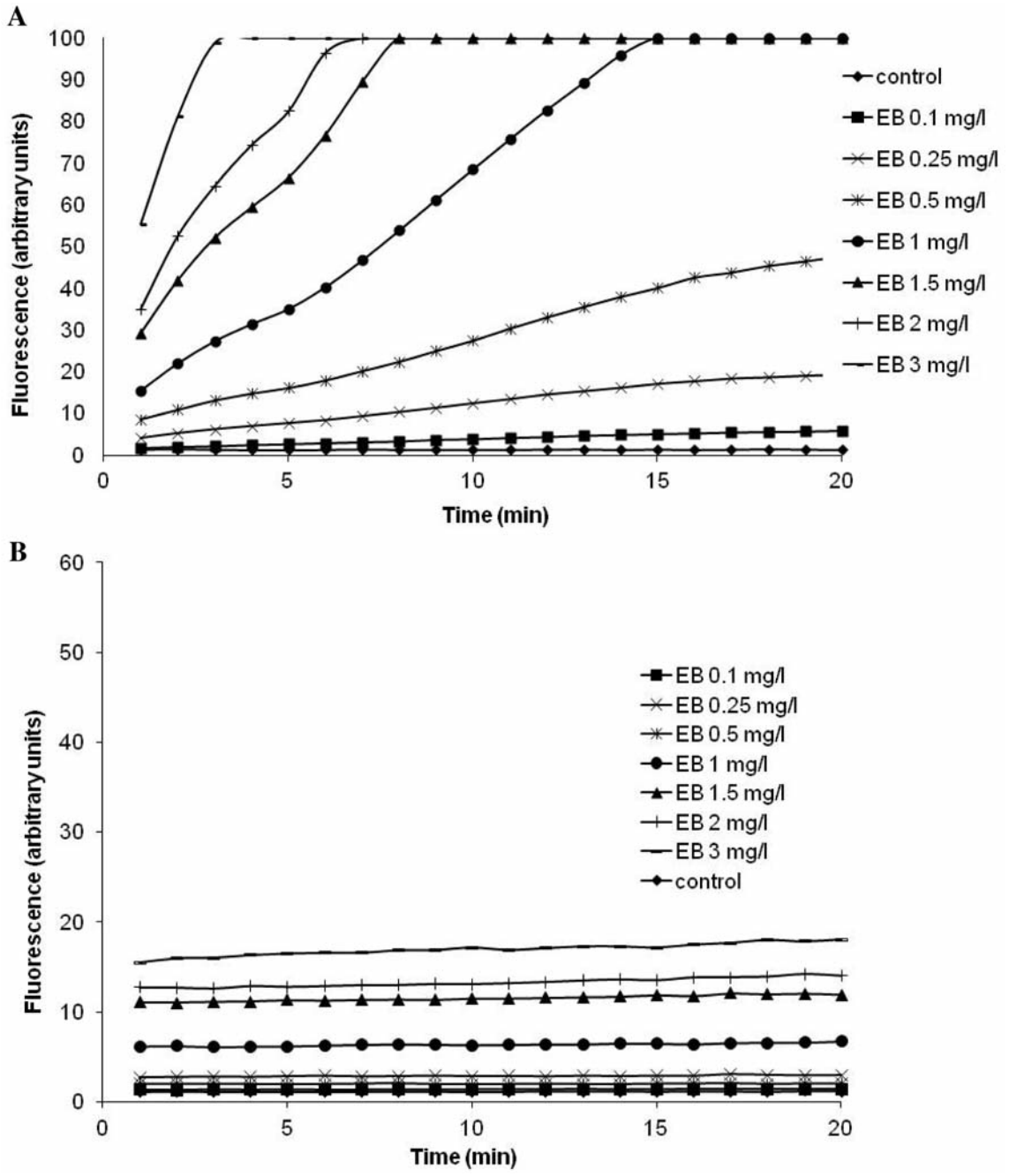


Figure 1. *Accumulation of EB bromide by Staphylococcus aureus ATCC 25923 (A) and Staphylococcus aureus HPV-107 (B) at increasing concentrations of ethidium bromide (EB). The fluorescence noted at 1 minute in (A) represents the level of fluorescence produced by the concentration of EB of the medium. This level of fluorescence remains unchanged in the absence of the bacterial cells. Figure 1B serves to demonstrate this observation. The degree of fluorescence present at the end of 1 minute in (B) is due to that produced by the concentration of the EB in the absence of cells. Because this level of fluorescence remains unchanged, the HPV-107 does not accumulate EB even with concentrations of EB as high as 3 mg/l.*

hydantoin compound promotes some accumulation of EB by the HPV-107 strain, it does so at a much higher concentration (200 mg/l), suggesting that there is some effect on the Qac efflux pump present in this strain. Nevertheless, the increase of fluorescence noted at the beginning of the assay is maintained. This suggests that the efflux of EB is taking place at a level of extrusion of EB that is fairly close to the maximum which the HPV-107 cell is capable of extruding. The observation that

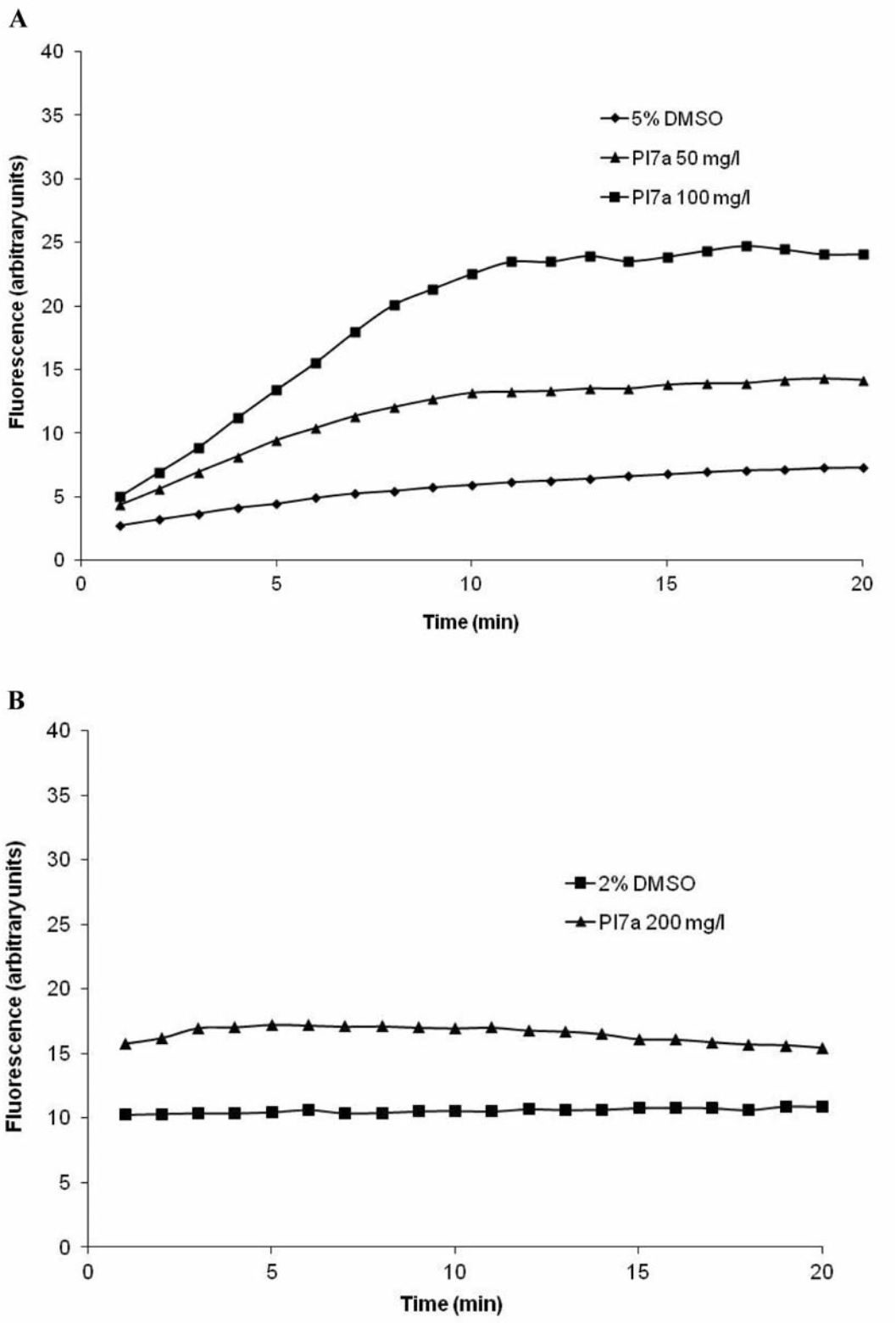


Figure 2. *Effect of hydantoin derivative PI7a on the accumulation of ethidium bromide (EB) by Staphylococcus aureus ATCC 25923 (A) and HPV-107 (B). In (A), fluorescence produced by 0.25 mg/l of EB has been deducted from each fluorescence value. In (B), fluorescence produced by 2.0 mg/l of EB has been deducted from each fluorescence value. Because above 200 mg/l the hydantoin tends to precipitate, the maximum concentration of hydantoin compound PI7a was used. Note that there is no change in the level of fluorescence during the assay period of 20 minutes.*

a much higher concentration of PI7a is needed to produce accumulation of EB by HPV-107 than that produced in the ATCC strain by the same compound is consistent with the expectation that if an agent is to inhibit a given efflux pump system, greater amounts of the agent are needed for the inhibition of an over-expressed efflux pump or one whose presence is in addition that of the intrinsic system (13, 14). The specific activity (SA) of the hydantoin derivatives evaluated in this study is presented in Table II. As noted from the data presented, with the exception of HY84, the activity of the hydantoins against the efflux pump system of the ATCC strain is considerably greater than that towards the HPV-10^7 strain. The hydantoins that had the highest SA against the efflux pump systems were evaluated for their ability to reduce or reverse resistance of each of the strains to oxacillin. Whereas none of the hydantoins reduced the MIC of oxacillin against the ATCC strain (MIC=0.125 mg/ml) hydantoin PI8a reduced the MIC of oxacillin against the HPV-107 strain from an initial level of 100 mg/l to 6 mg/l.

Discussion

The results obtained from this study indicate that whereas a number of compounds inhibited the efflux pump of the ATCC strain, these same compounds had a much less pronounced effect on the efflux pump of HPV-107 strain. However, whereas none of the compounds that had activity against ATCC strain were able to reduce the MIC of oxacillin against this strain, only one compound PI8a, containing two *p*-fluorophenyl moieties, significantly reduced the MIC of oxacillin against the HPV-107 strain. It is not surprising that some compounds that inhibit an intrinsic efflux pump of a bacterium do not reverse resistance to a given antibiotic, given that the efflux pump assay is conducted in minimal medium and over a short period of time, whereas the assay that evaluates a compound's ability to reduce or reverse resistance to a given antibiotic takes place in complete medium and over a period of 16 or more hours. The latter assay is known to provide the conditions needed for the inducement of genes that promote the overexpression of a given efflux pump system when the organism is placed under the stress created with exposure to an antibiotic (15-17). Consequently, the level of efflux pump activity of an intrinsic or overexpressed efflux pump may be readily affected in an assay that does not support genetic activity that results in the production of additional pump units. Nevertheless, if an inhibitor of a given efflux pump is to be pharmaceutically considered for further development, the fact that the hydantoin PI8a, in addition to inhibiting the Qac efflux pump expressed by the HPV-107 multidrug-resistant strain, also reduced the MIC to oxacillin, suggests that this non-toxic compound may have clinical potential for therapy of MRSA infection whose multidrug-resistant nature is due to overexpression of an efflux pump.

Table II. *Specific activity (SA) of hydantoin compounds on the accumulation of ethidium bromide by Staphylococcus aureus ATCC 25923 and Staphylococcus aureus HPV-107. The SA of each hydantoin on the efflux pump of the ATTC and HPV-107 strains was calculated by the formula presented in the Materials and Methods. The SA is defined as the relative fluorescence per 20 minutes/μmol of hydantoin compound. The higher the number, the greater the effect of the compound on the given efflux pump system. The SAs are listed in order of strength. Note, with exception of HY84, the SA of each hydantoin is far greater on the efflux pump system of the ATCC strain than on the HPV-107 strain.*

SA		SA	
S. aureus ATCC 25923		*S. aureus* HPV 107	
Compound	RFI/μmol	Compound	RFI/μmol
PI8a	165.3	HY83	44.7
PI6a	118.1	HY84	20.8
PI2a	113.0	PI3a	13.9
PI5a	108.8	PI4a	13.3
PI7a	100.6	PI6a	13.2
PI4a	97.7	PI7a	11.3
PI3a	96.9	PI2a	6.2
PI1a	94.6	PI8a	4.7
PI11a	77.5	MF8	2.9
PI10a	47.8	PI1a	2.6
HY83	43.6	KF4	1.1
MF8	32.4	PI5a	–0.5
KF4	31.8	PI9a	–0.6
PI9a	30.5	PI10a	–2.0
HY84	14.3	PI11a	–2.9
KF20	9.9	KF20	Not done

Acknowledgements

Partly supported by grants: 501/N-COST/2009/0 (K/PMN/000031), COST action BM0701. A. Dymek was supported by an STSM grant from COST ACTION BM0701 (ATENS). L. Amaral was supported by BCC grant SFRH/BCC/51099/2010 provided by the Fundação para a Ciência e a Tecnologia (FCT) of Portugal and PTDC/SAU-FCF/102807/2008. This work was supported by EU-FSE/FEDER-PTDC/BIA-MIC/105509/2008 and EU-FSE/FEDERPTDC/SAU-FCF/102807/2008 from the Fundação para a Ciência e a Tecnologia (FCT) of Portugal. G. Spengler was supported by TÁMOP-4.2.1/B-09/1/KONV-2010-0005 – Creating the Center of Excellence at the University of Szeged supported by the European Union and co-financed by the European Regional Fund.

References

1 Agarwal R, Burley SK and Swaminathan S: Structural analysis of a ternary complex of allantoate amidohydrolase from *Escherichia coli* reveals its mechanics. J Mol Biol *368(2)*: 450-463, 2007.

2 Syldatk C, May O, Altenbuchner J and Mattes R: Microbial hydantoinases–industrial enzymes from the origin of life? Appl Microbiol Biotechnol *51*: 293-309, 1999.

3 Weyand S, Shimamura T, Yajima S, Suzuki S, Mirza O, Krusong K, Carpenter EP, Rutherford NG, Hadden JM, O'Reilly J, Ma P, Saidijam M, Patching SG, Hope RJ, Norbertczak HT, Roach PC, Iwata S, Henderson PJ and Cameron AD: Structure and molecular mechanism of a nucleobase cation symport-1 family transporter. Science *322(5902)*: 709-713, 2008.

4 Spengler G, Handzlik J, Ocsovszki I, Viveiros M, Kieć-Kononowicz K, Molnar J and Amaral L: Modulation of multidrug efflux pump activity by new hydantoin derivatives on colon adenocarcinoma cells without inducing apoptosis. Anticancer Res *31*: 3285-3288, 2011.

5 Machado L, Spengler G, Evaristo M, Handzlik J, Molnár J, Viveiros M, Kieć-Kononowicz K and Amaral L: Biological activity of twenty-three hydantoin derivatives on intrinsic efflux pump system of *Salmonella enterica* enteritidis NCTC 13349. In Vivo *25*: 769-772, 2011.

6 Slabbekoorn M, Horlings HM, van der Meer JT, Windhausen A, van der Sloot JA and Lagrand WK: Left-sided native valve *Staphylococcus aureus* endocarditis. Neth J Med *68(11)*: 341-347, 2010.

7 Martins A, Couto I, Aagaard L, Martins M, Viveiros M, Kristiansen JE and Amaral L: Prolonged exposure of methicillin-resistant *Staphylococcus aureus* (MRSA) COL strain to increasing concentrations of oxacillin results in a multidrug-resistant phenotype. Int J Antimicrob Agents *29*: 302-305, 2007.

8 Couto I, Costa SS, Viveiros M, Martins M and Amaral L: Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide. Antimicrob Agents Chemother *62*: 504-513, 2008.

9 Costa SS, Ntokou E, Martins A, Viveiros M, Pouranas S, Couto I and Amaral L: Identification of the plasmid encoded qacA efflux pump gene in the methicillin-resistant *Staphylococcus aureus* (MRSA) strain HPV107, a representative of the MRSA Iberian clone. Int J Antimicrob Agents *36*: 557-561, 2010.

10 Kieć-Kononowicz K, Stadnicka K, Mitka A, Pekala E, Filipek B, Sapa J and Zygmunt M: Synthesis, structure and antiarrhythmic properties evaluation of new basic derivatives of 5,5-diphenylhydantoin. Eur J Med Chem *38(6)*: 555-566, 2003.

11 Kieć-Kononowicz K, Müller CE, Pekala E, Karolak-Wojciechowska J, Handzlik J and Łażewska D: Imidazo[2,1-*b*] thiazoles, imidazo[2,1-*b*]imidazoles and pyrrolo[1,2-*c*]imidazoles. Synthesis, structures and evaluation of benzodiazepine receptor binding. J Heterocycl Chem *39*: 243-253, 2002.

12 Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document *27(1)*: M100-S117, 2007.

13 Viveiros M, Rodrigues L, Martins M, Couto I, Spengler G, Martins A and Amaral L: Evaluation of efflux activity of bacteria by a semi-automated fluorometric system. Methods Mol Biol *642*: 59-72, 2010.

14 Martins A, Vasas A, Viveiros M, Molnar J, Hohmann J and Amaral L: Antibacterial properties of compounds isolated from *Carpobrotus edulis*. Int J Antimicrob Agents *37*: 410-414, 2011.

15 Spengler G, Rodrigues L, Martins M, McCusker M, Cerca P, Machado L, Costa, SS, Dokou E, Couto I, Viveiros M, Fanning S, Martins A, Molnar J and Amaral L: Genetic response of *Salmonella enterica* serovar Typhimurium to thioridazine rendering the organism resistant to the agent. Int J Antimicrobial Agents.

16 Viveiros M, Rodrigues l, Dupont M, Martins M, Couto I, Pagès JM and Amaral L: Antibiotic stress, genetic response and altered permeability of *E. coli* PLoS One *2*: 365, 2007.

17 Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, Molnar AM, Molnar J and Amaral L: Inducement and reversal of tetracycline resistance in *Escherichia coli* K-12 and the expression of proton gradient-dependent multidrug efflux pump genes. Antimicrobial Agents Chemother *49*: 3578-3582, 2005.

Received November 8, 2011
Revised December 13, 2011
Accepted December 14, 2011