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Jadwiga Handzlik, Ewa Szymańska, Sandrine Alibert, Jacqueline Chevalier, Ewa Otrębska, et al. Search for new tools to combat Gram-negative resistant bacteria among amine derivatives of 5-arylidenehydantoin. *Bioorganic and Medicinal Chemistry*, 2013, 21 (1), pp.135 - 145. 10.1016/j.bmc.2012.10.053 . hal-01425050

HAL Id: hal-01425050

<https://amu.hal.science/hal-01425050>

Submitted on 16 Jan 2017

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Search for new tools to combat Gram-negative resistant bacteria among amine derivatives of 5-arylidenehydantoin

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Keywords: Arylideneimidazolidine-2,4-dione Arylidenehydantoin, *Enterobacter aerogenes* Multidrug, resistance, Efflux pump, AcrAB-TolC

A series of amine-alkyl derivatives of 5-arylidenehydantoin 3–21 was evaluated for their ability to improve antibiotic effectiveness in two strains of Gram-negative *Enterobacter aerogenes*: the reference strain (ATCC-13048) and the chloramphenicol-resistant derivative overproducing the AcrAB-TolC efflux pump (CM-64). Three antibiotics, chloramphenicol, nalidixic acid and sparfloracin were used as markers of efflux pump activity. New compounds (5–16) were obtained within 3–4 step synthesis using Knoevenagel condensation, Mitsunobu reaction and microwave aided N-alkylation. Molecular modeling based structure–activity relationship (SAR) studies were performed. The most active compounds: (Z)-5-(4-(diethylamino)benzylidene)-3-(2-hydroxy-3-(4-(2-hydroxyethyl)piperazin-1-yl)propyl)imidazolidine-2,4-dione (14) and (Z)-5-(2,4-dimethoxybenzylidene)-3-(2-hydroxy-3-(isopropylamino)propyl)imidazolidine-2,4-dione (15) induced fourfold decrease of minimal inhibition concentration (MIC) of all tested antibiotics in the strain CM-64 overexpressing the AcrAB-TolC pump.

1. Introduction

Multidrug resistance (MDR) is a serious problem in therapy of bacterial,^{1–9} fungal¹⁰ and cancer diseases.^{11–13} In each case, one of the main mechanisms of MDR involves efflux pumps, for example, membrane transport proteins which are able to expel drugs or antibiotics before they can reach their intracellular targets. The MDR efflux pumps belong to transporter families, including two main superfamilies: major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) superfamily⁸ playing an important role in cancer MDR.^{11–13} Furthermore, two sub-classes of efflux transport proteins are described: the small multidrug resistance family (SMR) and the resistance/nodulation/division family (RND).⁷ The RND transporters are particularly widespread among Gram-negative bacteria as it is illustrated by the tripartite efflux system AcrAB-TolC in *Escherichia coli* or MexAB-OprM in *Pseudomonas aeruginosa* (the active pump component being AcrB or MexB, respectively).^{3,8}

With the continuous increase of reports describing MDR bacteria and the involvement of efflux pumps in clinical resistant isolates, strategies to annihilate the pump mechanism to preserve

an appropriate intracellular concentration of antibiotics are urgently needed.^{3,5} To this aim, different ways have been proposed: (i) inhibiting the activity of efflux pump by selective competition, (ii) blocking the membrane channel by original plugs or (iii) collapsing the energy driving force used by efflux mechanism.^{3,5} During last decade, new compounds, so called efflux pump inhibitors/modulators (EPIs), have been described for structural-activity studies on MDR mechanism at the molecular level and for their capacity to improve the activity of antibiotics and chemotherapeutics.^{3,5} During the search for new tools to combat MDR among bacterial pathogens, a special attention is paid to Gram-negative bacteria due to their double-membrane cells and the complex membrane barrier for antibiotic penetration corresponding to outer membrane structure. Among resistant Gram-negative bacteria, *Enterobacter aerogenes* is frequently described. This bacterium, responsible for hospital-acquired respiratory tract infections, exhibits a significant decrease of antibiotic susceptibility.² Studies on MDR mechanism in *E. aerogenes* indicated that tripartite efflux pump AcrAB-TolC is a major MDR system in clinical isolates.

Recent lines of evidence^{3–6} have described several groups of chemical compounds with EPI-properties in Gram-negative bacteria, including peptidomimetics, arylpiperazines, quinolines or pyridopyrimidines (Fig. 1a). For most of the compounds, however, a therapeutic usage as ‘adjuvant’ is rather impossible because of their toxicity and possible adverse side effects. Thus, a search for

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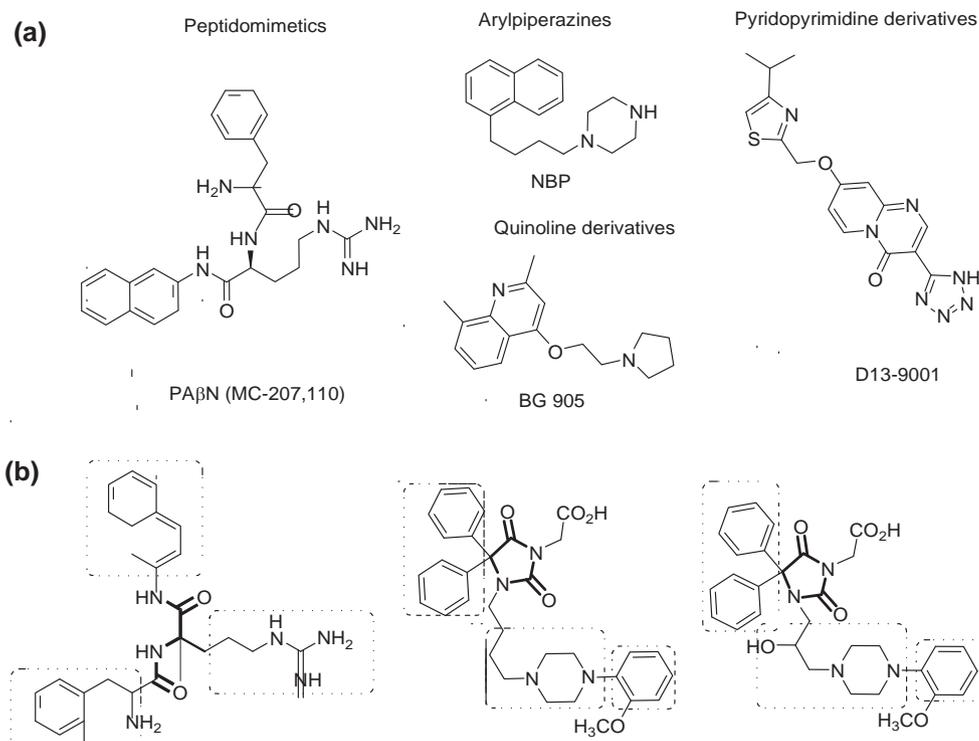


Figure 1. Structure of modulators of bacterial MDR-protein pump; a) potent efflux pump inhibitors; b) the recently found hydantoin derivatives with moderate activity (1 and 2); structural similarities to the reference in PABN (peptide cores in bold style, marked aromatic and basic fragment).^{5,9}

new EPIs in different chemical groups is a current question of medicinal chemistry.

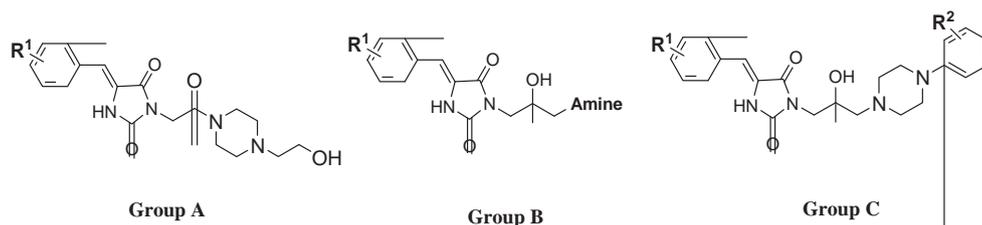
On the other hand, hydantoin derivatives, that are in the area of interest in our research group, display various biological actions, particularly GPCR-ligand properties^{14–16} and/or hypotensive, anti-arrhythmic or anticonvulsant activities in both in vitro and in vivo assays.^{17–19} Our previous studies,⁹ focused on hydantoin derivatives with amine-alkyl substituents at position N1, showed that the hydantoin could be a new appropriate scaffold to investigate relationships between molecular structures and their influence on antibiotic susceptibility. Indeed, the hydantoin skeleton could be viewed as a cyclic peptide sequence similar to the open sequence located in the central part of PabN structure (Fig. 1b). This semi-rigid structure allows to modify the nature and the position of substituents and facilitates molecular modeling to guide rational synthesis. The 5,5-diphenylhydantoin compounds described previously⁹ displayed weak or moderate ability to increase the activity of nalidixic acid in *E. aerogenes*. Among 28 tested compounds with the same scaffold, only two 2-methoxyphenylpiperazine derivatives with acetic acid motif (compounds 1 and 2, Fig. 1b) displayed significant anti-MDR effect at a dose range close to that of PABN. On the basis of those not satisfying results, in the present work we decided to introduce significant changes into the structure of hydantoins comparing to the initial series.⁹ In this context, chemical modifications of the 5,5-diphenylhydantoins were designed to improve activity by: (1) a replacement of 5,5-diphenyl-substituents with 5-arylidene one, (2) an exchange of position of amine-alkyl substituents from N1 into N3 or (3) an introduction of carbonyl or hydroxyl motif into the alkyl chain (Table 1). Thus, the present studies concern nineteen amine derivatives of 5-arylidenehydantoin 3–21 (Table 1). Synthesis of new compounds and microbiological assays to evaluate their anti-MDR properties in Gram-negative bacteria were performed. Molecular modeling aided structure–activity relationship analysis was carried out.

2. Results and discussion

2.1. Synthesis

The presented groups of 5-arylidenehydantoin derivatives 3–21 were divided into three sub-groups, including (A) amide hydroxyethylpiperazine derivatives 3 and 4, (B) derivatives of secondary- or tertiary non-aromatic amine 5–16, and (C) derivatives of phenylpiperazine 17–21 (Table 1). Synthesis routes of the arylidenehydantoins (3–21) are shown in the Schemes 1 and 2. Synthesis of compounds 3, 4 and 17–21 is described elsewhere.^{19,24} Compounds 5–9 and 11–16 were synthesized within three-step synthesis starting from hydantoin (Scheme 1) that was condensed with suitable aromatic aldehydes to give 5-arylidenehydantoins 23–29. The condensations went as typical Knoevenagel reactions in basic conditions^{19,24–26} by the use of sodium acetate in acetic acid (23–25, 27–29)^{19,24} or equimolar sodium carbonate and alanine in water (26).²⁶ The resulted 5-arylidenehydantoins (23–29) occurred at their (Z)-configuration, which is preferable in the case of Knoevenagel condensation between N1-unsubstituted hydantoin and the considered benzaldehydes.^{27–29} In the next step, the arylidenehydantoins 23–29 were N-alkylated at position 3 within Mitsunobu reactions to give oxiran derivatives 30–36. The reactions were performed using racemate of oxiran-2-ylmethanol (1.5 equiv), triphenylphosphine (1.0 equiv) and diethyl azodicarboxylate (1.0 equiv) in dry DMF²⁴ (Scheme 1). In the last step, the oxiran derivatives 31–35 were used as alkylating agents to react with primary- or secondary amines. In the case of compound 33, the alkylation was performed in solvent-free conditions under microwave irradiation^{14,18} (Scheme 2), what allowed to obtain 2-hydroxypropyl amine derivatives 5–9 in 16–28 min. Synthesis of compounds 11–16 was performed in methanol. The oxiranes 31, 32, 34 or 35 were refluxed in excess of a suitable amine (4.0 equiv) for 3 h. Compound 10, possessing free piperazine terminated

Table 1
Structure of the tested derivatives of 5-arylidenehydantoin 3–21



Compound	Group	R ¹	R ²	Amine
3	A	H	—	—
4	A	4-Br	—	—
5	B	4-MeO	—	
6	B	4-MeO	—	
7	B	4-MeO	—	
8	B	4-MeO	—	
9	B	4-MeO	—	
10	B	4-MeO	—	
11	B	4-Cl	—	
12	B	4-Cl	—	
13	B	4-Br	—	
14	B	4-NEt ₂	—	
15	B	2,4-diMeO	—	
16	B	2,4-diMeO	—	
17	C	H	2-MeO	—
18	C	4-Cl	2-MeO	—
19	C	4-Cl	2-EtO	—
20	C	3,4-diMeO	2-MeO	—
21	C	3,4-diMeO	H	—

fragment at position 3 of hydantoin, was obtained by acidic hydrolysis of acetyl piperazine derivative 8 in 15% HCl (Scheme 2).

2.2. Pharmacology

Compounds 3–21 were tested in microbiological assays for their ability to increase antibiotics effectiveness in two strains of *E. aerogenes*: a reference strain (ATCC-13048) and the derivative CM-64 which over-produces the AcrAB-TolC efflux pump.

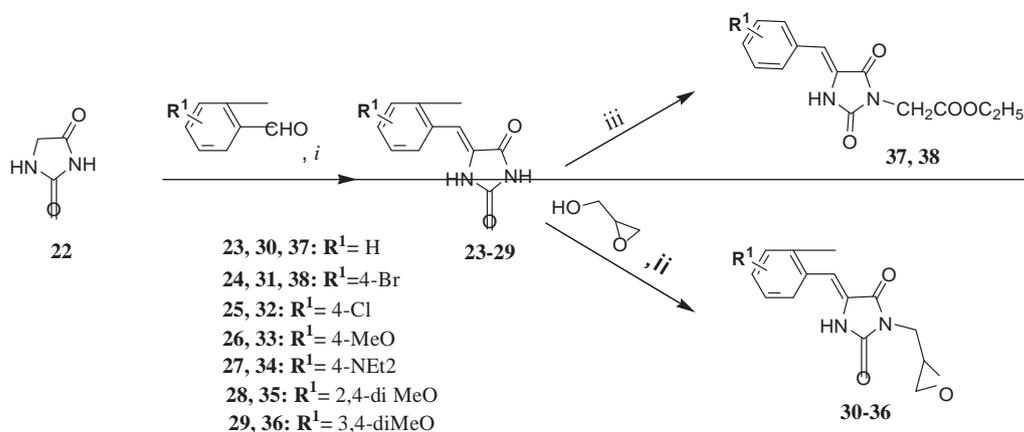
2.2.1. Intrinsic antibacterial activity

In the first step of the biological assays, direct antibacterial activity of the hydantoin derivatives was evaluated. Minimal inhibitory concentration (MIC) of the compounds 3–21 for both strains was determined (Table 2). The tested compounds displayed very

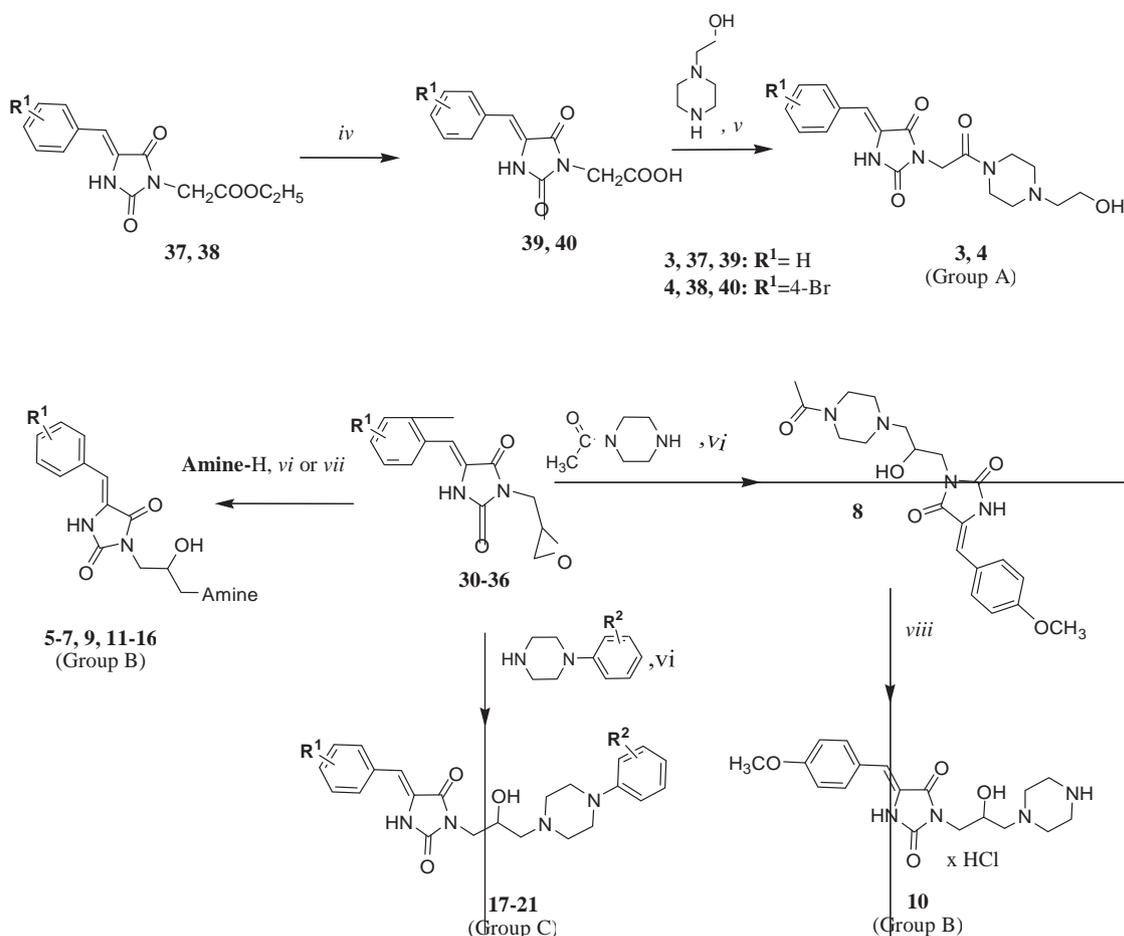
weak direct antibacterial activities. The lowest MICs observed for hydroxyethyl piperazine derivatives 3, 14 and 16 were in the range of 125 μ M whereas all derivatives of 4-methoxybenzylidenehydantoin (5–10) did not inhibit the growth of both strains of *E. aerogenes* at 2 mM. This low intrinsic effect allowed us to test these molecules in combination with usual antibiotics.

2.2.2. Influence on antibiotic MIC

In the next step of the microbiological assays, the compounds 3–21 were tested for their ability to influence the activity of the three following antibiotics: nalidixic acid (NAL), sparfloxacin (SFX) and chloramphenicol (CHL) in the tested bacteria strains. All the compounds have been assayed at a concentration that have no intrinsic effect, usually $\frac{1}{3}$ of their respective MIC (Table 2), in combination with usual antibiotics and a control was systematically



Scheme 1. Synthesis of intermediates 23–38: (i) Knoevenagel condensation $\text{CH}_3\text{COONa}/\text{CH}_3\text{COOH}$ ^{19,24} or $\text{Na}_2\text{CO}_3/\text{alanine}/\text{H}_2\text{O}$ ²⁶; (ii), DIAD or DEAD, TPP, dry DMF, 0 °C;²⁴ (iii) $\text{ClCH}_2\text{COOEt}$, K_2CO_3 , TEBA, acetone.^{14,18}



Scheme 2. Synthesis of products 3–21: (iv) $\text{EtOH}/\text{H}_2\text{O}$, NaOH ; (v) TEA, DMF, BOP;¹⁹ (vi) microwave irradiation; (vii) MeOH, boiling temp, 3 h; (viii) H_2O , H^+ .

carried out without antibiotic. For most of the compounds, the used concentration (63 μM , 6MIC/4) was in the range of the active concentration used for PabN (50 μM). The concentration was established for the tested compounds as a suitable one to compare their activities to those of previous hydantoin tested at the concentration of 63 μM , as well.⁹ Weakly active phenylpiperazine compounds 17–21 were tested at their concentration (100–200 μM) which ensured a lack of a direct antibacterial effect. The

‘activity gain’ (A)⁹ was calculated according to the equation presented in Figure 2. The tested compounds showed moderate or low chemosensitizing properties when conjointly added to each antibiotic, displaying the highest activity gains at the range of 4.

In the case of nalidixic acid assay (Fig. 2a), only twofold decrease of MIC values was observed for relatively most efficient compounds (6, 14, 15, 17, 18 and 21) in reference strain of *E. aerogenes*, whereas eight arylidene hydantoin (3, 4, 12, 14–16, 18 and 19) decreased

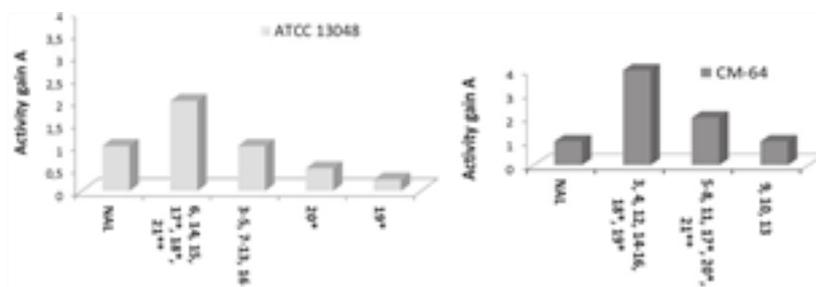
Table 2
Intrinsic antibacterial activity for compounds 3–21, tested in two strains of *E. aerogenes*, ATCC 13048 (reference) and the derivative strain CM-64 (over-producing AcrAB-TolC)

Compound	MIC (mM) EA ATCC 13048	MIC (mM) EA CM-64
4, 13	>1.25	>1.25
5–10	>2	>2
20	1	1
17–19	0.5	1
21	0.5	0.5
11, 12, 15	0.25	0.25
3, 14, 16	0.125	0.25
NAL ^a	0.008	0.128
SFX ^b	6 1 10 ⁵	0.001
CHL ^c	0.004	0.256
PAbN	5	5

^a Nalidixic acid.
^b Sparfloxacin.
^c Chloramphenicol.

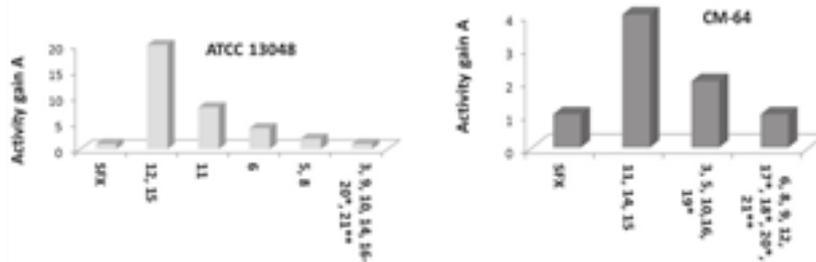
MIC of this antibiotic in fourfold in the efflux over-producer CM-64. A slight increase of efficacy of nalidixic acid (A = 2) in CM-64 was observed for eight arylidenehydantoin derivatives 5–8, 11, 17, 20 and 21, and four compounds (4, 9, 10 and 13) did not cause any increase. In the case of assays with sparfloxacin, four compounds (6, 11, 12 and 15) significantly decreased MIC of this antibiotic in the reference strain (ATCC 13048). Particularly, compounds 12 and 15 caused effect higher than that of PAbN in the same strain (Fig. 2b). Compounds 11, 15 and the diethylaminobenzylidene derivative 14 showed fourfold decrease of sparfloxacin in the efflux over-producer CM-64. Twofold increase of sparfloxacin efficacy was observed in the presence of hydantoin derivatives 3, 5, 10, 16 or 19. The rest of the tested compounds (6, 8, 9, 12, 17, 18, 20 and 21) did not influence the MIC of sparfloxacin during the assay. Results for the assays with chloramphenicol (Fig. 2c) indicated that only two compounds (6 and 19) slightly reduced MIC of this antibiotic in the reference strain, but four compounds (11, 14–16) caused fourfold- and six compounds (3, 6,

(a) Nalidixic acid



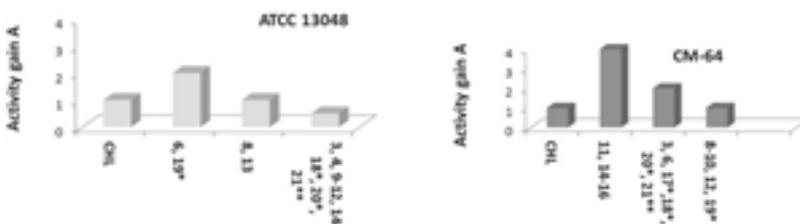
Activity gains (A) for PAbN, at concentration of 50 μM, were as follows: 64 (ATCC 13048), 128 (CM-64).

(b) Sparfloxacin



Activity gains (A) for PAbN, at concentration of 50 μM, were as follows: 8 (ATCC 13048), 32 (CM-64).

(c) Chloramphenicol



Activity gains (A) for PAbN, at concentration of 50 μM, were as follows: 2 (ATCC 13048), 64 (CM-64).

$$A = \frac{\text{MIC of antibiotic in absence of a compound (3-21)}}{\text{MIC of antibiotic in presence of the compound (3-21)}}$$

Figure 2. The influence of the compounds 3–21 on minimal inhibitory concentration (MIC) of (a) nalidixic acid (NAL), (b) sparfloxacin (SFX) and (c) chloramphenicol (CHL), tested in two strains of *E. aerogenes*, ATCC 13048 (reference) and CM-64 (with over-production of AcrAB-TolC). Compounds tested at concentration of 63 μM; compound tested at 200 μM; compound tested at 100 μM.

Table 3
Effect of hydantoin derivatives (3–21) on the antibiotic susceptibility of *E. aerogenes* ATCC-13048 and CM-64 strains

Compound ^a	EA ATCC-13048			EA CM-64		
	NAL MIC (1g/mL)	SFX MIC (1g/mL)	CHL MIC (1g/mL)	NAL MIC (1g/mL)	SFX MIC (1g/mL)	CHL MIC (1g/mL)
No	8	0.06	4	128	1	512
3	8	0.06	8	32	0.5	256
4	8	^b	8	32	^b	^b
5	8	0.03	^b	64	0.5	^b
6	4	0.015	2	64	1	256
7	8	^b	^b	64	^b	^b
8	8	0.03	4	64	1	512
9	8	0.06	8	128	0.5	512
10	8	0.06	8	128	0.25	512
11	8	0.007	8	64	1	128
12	8	0.003	8	32	^b	512
13	8	^b	4	128		^b
14	4	0.06	8	32	0.25	128
15	4	0.003	8	32	0.25	128
16	8	0.06	8	32	0.5	128
17	4	0.06	8	64	1	256
18	4	0.06	8	32	1	256
19	32	0.06	2	32	0.5	512
20	16	0.06	8	64	1	256
21	4	0.06	8	64	1	256

^a Compounds concentrations according to the Figure 2.

^b Compounds which induced aggregates of bacteria in the presence of sparfloxacin or chloramphenicol. These aggregates did not allow MICs measurement.

17, 18, 20, 21) caused twofold reduction of chloramphenicol MIC in the efflux over-producer CM-64.

The results obtained during the assays with nalidixic acid and chloramphenicol indicated that ability of the hydantoin derivatives (3–21) to improve antibiotic efficacy is distinctly higher in CM-64, the strain of *E. aerogenes* which over-produces AcrAB-TolC efflux pump, than that in the reference strain (Table 3). This suggests that their way to modulate the bacterial resistance is connected to the tripartite efflux pump action. Four compounds (6, 11, 12 and 15) involve some additional anti-MDR mechanism distinct from AcrAB-TolC pump as they cause decrease of MIC of sparfloxacin significantly higher in the reference strain of *E. aerogenes*. Particularly, it applies to compound 12 while the chemosensitizer potency of compounds 11 and 15 in CM-64 is noticeable as well.

An analysis of direct MIC of the tested compounds in comparison to their properties to enhance antibiotics indicate that the most active compounds 14 and 15 displayed relatively higher intrinsic antibacterial action (MIC = 250 **IM**). The compounds were tested at 1/4 of the MIC to ensure a lack of a direct antibacterial effect. As a role of drug-hydantoin combinations in the chemosensitizer action is concerned, the combination with nalidixic acid seems to be superior. Eight members of the tested population (3, 4, 12, 14–16, 18, 19) generated statistically different values of activity (A) comparing the over-producing MDR efflux pump strain (CM-64) to the wild one (ATCC 13048), significantly higher in the case of CM-64. The weakest hydantoin-drug collaboration was observed during the assays with sparfloxacin in the strain CM-64. Only one compound, the diethylaminobenzylidene derivative 14, generated statistically different higher A value for the strain over-producing MDR efflux pump AcrAB-TolC. In contrary, four compounds (12, 15 > 11 > 6) caused statistically different stronger increase of sparfloxacin action in the reference strain ATCC 13048.

The results obtained within the assays using strain CM-64 allow to divide the series of hydantoin derivatives (3–21) into following classes of potential efflux pump inhibitors: (I) the promising compounds which caused fourfold decrease of MIC of at least one antibiotic (3, 4, 11, 12, 14–16) at the lower dose (63 **IM**); (II) compounds with slight efflux-modulator properties (5–8, 10, 17–21) causing the twofold decrease at lower concentration or fourfold one at the higher concentrations (100–200 **IM**); and (III)

inactive compounds showing no effect in the efflux pump over-producing strains (9 and 13). From the effect obtained on CM-64 (Table 3), we can conclude that 14 and 15 were the more attractive molecules.

2.3. Molecular modeling

Analysis of structural features and structure–activity relationship for the set of the obtained compounds was based on their low energy conformations, calculated for a free base form of 3–21. On the basis of various lines of evidence including crystallographic studies for our previous 5-arylidenehydantoins,^{20–24,27–29} only (Z)-configuration was considered for whole investigated population. Our previous investigations performed for the arylpiperazines 17–21 showed that in case of molecules at the global minimum energy point the absolute configuration of the secondary alcohol carbon almost did not influence on calculated distances between crucial pharmacophoric elements.²⁴ Thus, this time we limited our investigations to one enantiomer with the (S)-configuration. The obtained lowest energy conformations for compounds 3–21 are presented in Figure 3. The superimposition of one of the most active chemosensitizer 14 with the rest of the tested population (3–13 and 15–21) is shown in respect to the three structural categories A–C.

From the structural point of view, all the studied molecules consist of a flat moiety of hydantoin, linked by methyldene bridge with a (un)substituted phenyl ring from one side, and by flexible hydroxypropyl or ethanone chain with a secondary or tertiary amine group from the other. In the energetic global minimum all the compounds adopted an extended conformation with the arylidene phenyl ring rotated either by c.a. 57° or \tilde{y} 57° with reference to the imidazol ring plane, regardless of the amine fragment (Fig. 3). It should be noticed here that both positions of the phenyl ring were almost equivalent energetically and differed from each other with not more than 0.05 kJ/mol.

2.4. Structure–activity relationship

The arylidenehydantoin derivatives (3–21) differ within three structural fragments, what gives an opportunity to evaluate an

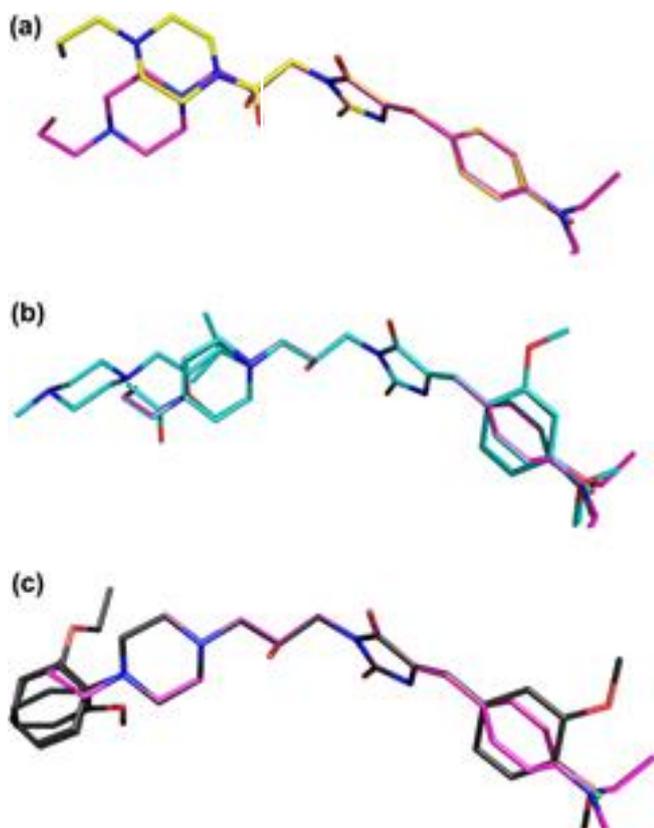


Figure 3. Molecular modeling results. The superimposition of the most active chemosensitizer 14 (purple) with the rest of the compounds in respect to the three structural groups: (a) compounds of the group A (yellow); (b) compounds of the group B (blue); (c) compounds of the group C (black). All conformations represent the global energy minimum point. The hydrogen atoms are not shown.

influence of the fragments on the compound properties to restore a susceptibility to investigated antibiotics. Considering an influence of the amine terminated fragments, non-aromatic amines (group A and B, Table 1) seem to be more profitable than phenylpiperazine (group C). Among phenylpiperazine derivatives (17–21), only two compounds (18 and 19) caused fourfold decrease of one antibiotic (NAL) but their action was identified at higher doses (100 μ M) than that of corresponding non-aromatic amine derivatives (Table 3, Fig. 2). Two non-aromatic amine moieties seem to be beneficial,

hydroxyethylpiperazine (3, 4, 12, 14 and 16) and isopropylamine (11 and 15). The hydroxyethylpiperazine fragment is particularly favorable to improve the antibacterial effect of nalidixic acid, whereas a role of this fragment is shared with isopropyl moiety in the case of assays with sparflaxacin and chloramphenicol. The compounds 14 and 15 may be identified as the most active chemosensitizers of the resistant *E. aerogenes* because of their fourfold MIC reduction for all the tested antibiotics (Table 3). The compounds contain an aromatic arylidene fragment which is significantly activated by electron donating substituents as follows: a strong activating group of diethylamine at position para (14) or two moderate activating groups of 2,4-dimethoxy substituents (15). Considering the influence of substitution at the arylidene aromatic rings on the tested biological activities, it can be noted that 4-diethylamine- (14), 2,4-dimethoxy- (15 and 16) and 4-chloro- (11 and 12) substituents are the most favorable. Furthermore, the substituent-free benzylidene fragment (3) seems to be more profitable than the 4-methoxybenzylidene (6–10). The role of 4-bromide substituent is hard to evaluate since the compounds possessing this fragment (4, 13) caused an aggregation allowing to test them only with nalidixic acid. The 4-chlorobenzylidene fragment, present in compounds 11 and 12, improved efficacy of sparflaxacin similarly- (11) or higher (12) than PAbN in the reference strain of *E. aerogenes*. This suggests that the p-Cl substituent may promote some unknown mechanism of anti-MDR action which disappears when tripartite efflux pump AcrAB-TolC is the predominant resistance mechanism, as in strain CM-64. A role of linkers between amine and hydantoin is difficult to explain on the basis of the obtained results. Although the compounds possessing acyl linker (3 and 4) were relatively less active than compounds with the hydroxypropyl one (11, 14–16), the structural differences of the both groups involves other fragments (e.g., substituents at arylidene moieties).

Chemical properties of the most active compounds (14, 15), assessed basing on their 3D-structure, allow to propose pharmacophore features which may be responsible for chemosensitizing action on the MDR pump AcrAB-TolC, observed among the tested hydantoin (Fig. 4). It could be seen that an arylidene moiety at position 5 (AR) as well as a basic positive ionizable area linked to position 3 of the hydantoin (PIs) are crucial for the activity. A non-aromatic hydrophilic substituent at the positive ionizable area (HYL) seems to improve reversal-MDR activity of compounds, particularly in the case of nalidixic acid, whereas electron donating substituents in para- and ortho-position of the aromatic ring (ED) enable to develop the activity for all tested antibiotics. An aromatic

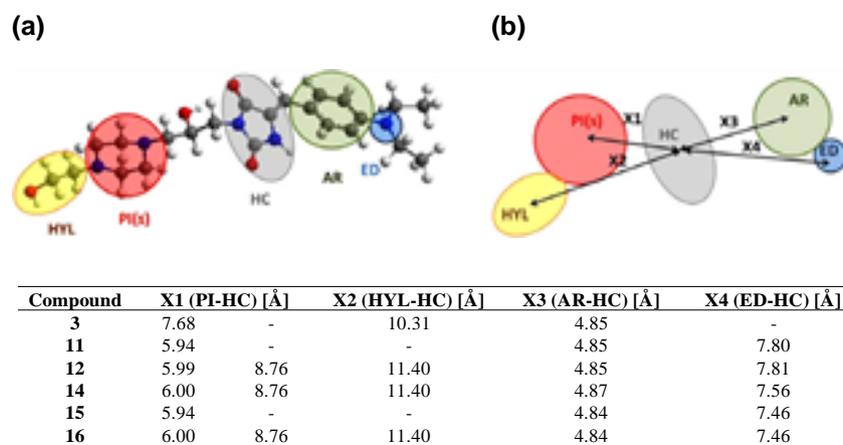


Figure 4. Pharmacophore features proposed based on properties of a most promising compound (14); (a) HC-hydantoin core, AR-aromatic ring, ED-electron donating substituent at aromatic ring, PI(s)-area with positive ionizable center(s), HYL-hydrophilic non-aromatic substituent; (b) distances from the center of hydantoin core to PI (X1), HYL (X2), AR (X3) and ED (X4) estimated for the most promising compounds 3, 11, 12, 14–16.

substituent at the piperazine area PI can be considered as an undesirable feature decreasing anti-MDR properties of the compounds. This can underline a role of both, HYL and PIs, since the hydrophobic aromatic substituent at piperazine nitrogen drastically changes its basic properties and causes a huge increase of lipophilicity of the whole substituent at position 3 of the hydantoin.

Molecular modeling calculations allowed to estimate topology of four pharmacophoric areas (PI, HYL, AR, ED) of the promising compounds (3, 11, 12, 14–16) in respect to the centre (HC) of the hydantoin scaffold (Fig. 4). Analysis of the calculated distances (X1–X4) indicated that the rotation of the arylidene phenyl ring (AR) by 57° or ̳57° with reference to the imidazol ring (HC) did not affect the distance X3 between both fragments. The distance was in range of 4.84–4.87 Å (Fig. 4) for all the tested compounds (3–21, Fig. 4). Only small differences can be noticed also in the case of the X1 value, expressed as a distance between the hydantoin ring and the closest basic positive ionizable centre (PI). Superimposition of 14 with the low-energy poses of compounds from coming B or C groups shows overlapping of the basic nitrogen atoms in piperidine/morpholine/piperazine as well as isopropylamine fragments. In the first case (10, 12, 14, 16, 17–21) the X1 distance was 6.00 Å (8.72–8.76 Å for the second basic nitrogen atom, if possible), while this distance was around 5.94 Å for isopropyl derivatives 11, 13 and 15. In the case of piperazinamides 3 and 4 from the group A, the distance X1 was extended to 7.68 Å (Fig. 3b).

3. Conclusions

In summary, the work contributes to the search of new tools to combat multidrug resistant bacteria among chemical compounds possessing hydantoin scaffold. From our previous studies,⁹ a new series of N3-aminealkyl arylidenehydantoin derivatives was designed, synthesized and evaluated for their ability to improve antibiotic efficacy in Gram-negative E. aerogenes bacteria over-producing AcrAB-TolC efflux pump. The SAR analysis was performed on basis of the microbiological assays and the molecular modeling calculation. In comparison to the previous series of N1-aminealkyl derivatives of 5,5-diphenylhydantoin,⁹ the line of chemical modifications of hydantoin described here (3–21) seems to be more favorable as it gave a higher number of new compounds displaying chemosensitizing action on the MDR bacteria. Seven of the tested compounds (3, 4, 11, 12, 14–16), used in the concentration closed to the active dose of PAbN, displayed significant four-fold increase of antibacterial activity of nalidixic acid (3, 4, 12, 14–16), chloramphenicol (11, 14–16) and/or sparfloxacin (11, 14, 15). The general SAR derived from the studies indicates that an amphiphilic character of the compounds is beneficial for the expected chemosensitizing action on AcrAB-TolC, what can be expressed by 'antipodal' positions of both, the hydrophilic basic amine fragment and the hydrophobic aromatic one, in the case of the most active compounds. The SAR study also demonstrates that strong electron-donor properties of substituent(s) at the arylidene aromatic ring, that is seen in the case of (Z)-5-(4-(diethylamino)benzylidene)-3-(2-hydroxy-3-(4-(2-hydroxyethyl)piperazin-1-yl)propyl)imidazolidine-2,4-dione (14) and (Z)-5-(2,4-dimethoxybenzylidene)-3-(2-hydroxy-3-(isopropylamino)propyl)imidazolidine-2,4-dione (15), could be responsible for similar chemosensitizing properties observed in the assays with different antibiotics (NAL, CHL, SFX) in the strain over-producing MDR efflux pump AcrAB-TolC.

Although the studies gave a promising group of potential MDR efflux pump inhibitors, the most active compounds (14 and 15) displayed only moderate chemosensitizing properties, lower than that of peptidomimetics (PAbN). Thus, further modifications within the group of hydantoin derivatives are necessary to improve their

activity. Results of the comprehensive studies performed here will be a base for rational design of new MDR efflux pump inhibitors possessing hydantoin scaffold. The most active compounds, detected here, will be investigated using other bacterial species and antibiotic classes to understand their exact effect on various bacterial mechanisms of resistance and their precise target, a special attention will be paid for tripartite AcrAB-TolC efflux pump described in the MDR Enterobacterial isolates.

4. Experimental

4.1. Chemistry

¹H NMR spectra were recorded on a Varian Mercury VX 300 MHz PFG instrument (Varian Inc., Palo Alto, CA, USA) in CDCl₃ or DMSO-d₆ at ambient temperature using the solvent signal as an internal standard. IR spectra were recorded on a Jasco FT/IR-410 apparatus using KBr pellets and are reported in cm⁻¹. Thin-layer chromatography was performed on pre-coated Merck silica gel 60 F₂₅₄ aluminium sheets, the used solvent systems were (I) CH₂Cl₂/acetone 17:3; CH₂Cl₂, (II) CH₂Cl₂/acetone 19:1; (III) CHCl₃/i-PrOH/NH₃ 9:11:3; (IV) MeOH/acetone/benzene 1:1:1; (V) toluene/acetone/methanol 15:5:1. Melting points were determined using Mel-Temp II apparatus and are uncorrected. Elemental analyses were within ±0.4% of the theoretical values unless stated otherwise. Syntheses under microwave irradiation were performed in household microwave oven Samsung M1618. Synthesis of compounds 3, 4, 17–21, 23–29, 31, and 34–38 has been described elsewhere.^{19,24}

4.1.1. General procedure for preparation of 5-arylidene-3-(oxiran-2-ylmethyl)imidazolidine-2,4-diones (30, 32 and 33)

5-Arylideneimidazolidine-2,4-dione (10 mmol), oxiran-2-ylmethanol (15 mmol, 1.11 g) and triphenylphosphine (10 mmol, 2.62 g) were added to 50 mL of dry DMF at 0 °C. The mixture was stirred on the ice-bath until the reactants were totally dissolved (20 min). A solution of diethyl azodicarboxylate DEAD (10 mmol, 1.74 g) in dry DMF (10 mL) was added to the mixture dropwise for 45 min and stirring was continued at room temperature for further 3 h. Then, the mixture was poured into 100 mL of cold water to precipitate and left at 0–4 °C overnight. After filtration, pure product was obtained from precipitate by column chromatography with CH₂Cl₂/acetone and additional crystallization with EtOH or MeOH.

4.1.1.1. (Z)-5-benzylidene-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione (30). (Z)-5-benzylideneimidazolidine-2,4-dione 23 (1.88 g) was used. Crystallization with isopropanol gave white crystals of 30 (200 mg, 0.82 mmol, 8.2%), mp: 170–172 °C, R_f (I): 0.74. Anal. Calcd for C₁₃H₁₂N₂O₃: C, 63.93; H, 4.95; N, 11.47. Found: C, 63.85; H, 5.10; N, 11.04. ¹H NMR (CDCl₃) δ (ppm): 2.69 (dd, J₁ = 4.87 Hz, J₂ = 2.56 Hz, 1H, N3-CH₂CHCH_a(H)), 2.81–2.84 (m, 1H, N3-CH₂CHCH_b(H)), 3.22–3.28 (m, 1H, N3-CH₂CHCH₂), 3.75 (dd, J₁ = 14.36 Hz, J₂ = 4.87 Hz, 1H, N3-CH_c(H)), 3.90 (dd, J₁ = 14.36 Hz, J₂ = 5.13 Hz, 1H, N3-H_d(H)), 6.78 (s, 1H, CH@C), 7.35–7.48 (m, 5H, Ar-2,3,4,5,6-H), 8.37 (s, 1H, N1H). IR KBr (cm⁻¹): 3276 (N1H), 1764 (C2@O), 1711 (C4@O), 1656 (CH@C), 1573 (Ar).

4.1.1.2. (Z)-5-(4-chlorobenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione (32). (Z)-5-(4-chlorobenzylidene)imidazolidine-2,4-dione 25 (2.23 g) was used. Crystallization with isopropanol gave white crystals of 33 (750 mg, 2.6 mmol; 26.9%), mp: 207–212 °C, R_f (I): 0.67. Anal. Calcd for C₁₃H₁₁ClN₂O₃: C, 56.03; H, 3.98; N, 10.05. Found: C, 56.20.85; H, 4.03; N, 10.01. ¹H

NMR (CDCl₃) d (ppm): 2.69 (dd, J₁ = 4.87 Hz, J₂ = 2.57 Hz, 1H, N3-CH₂CHCH_a(H)), 2.83–2.86 (m, 1H, N3-CH₂CHCH_b(H)), 3.23–3.29 (m, 1H, N3-CH₂CHCH_c), 3.76–3.89 (m, 2H, N3-CH₂), 6.72 (s, 1H, CH@C), 7.38–7.44 (m, 4H, Ar-2,3,5,6-H), 8.86 (s, 1H, N1H).

4.1.1.3. (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione (33). (Z)-5-(4-methoxybenzylidene)imidazolidine-2,4-dione 26 (2.18 g) was used. Purification by column chromatography gave white crystals of 33 (1.34 g, 4.8 mmol, 49%), mp: 176–179 °C, R_f (II): 0.59. Anal. Calcd for C₁₄H₁₄N₂O₄: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.44; H, 5.25; N, 10.28. ¹H NMR (DMSO-d₆) d (ppm): 2.52–2.55 (m, 2H, CH₂O), 3.15–3.17 (m, 1H, CHO), 3.63–3.65 (d, J = 4.87 Hz, 2H, N3-CH₂), 3.79 (s, 3H, OCH₃), 6.52 (s, 1H, CH@C), 6.95–6.98 (m, 2H, Ar-3,5-H), 7.60–7.63 (m, 2H, Ar-2,6-H), 10.72 (s, 1H, N1H).

4.1.2. General procedure for preparation of hydrochloride form of amine derivatives of 5-arylideneimidazolidine-2,4-diones (5–7)

(Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione (33) (1.6–3.0 mmol) and appropriate amine (2.8–6.0 mmol) were irradiated in a standard household microwave oven at various power (600–750 W) and time intervals (16–24 min) of irradiation for each prepared compound (5–7), respectively. The reaction progress was controlled with TLC (toluene (15):acetone (5):methanol (1)). After irradiation, the glassy residue was crystallized and saturated with gaseous HCl to give a pure product in hydrochloride form. The precipitate of pure product (5–7) was separated by filtration.

4.1.2.1. (Z)-5-(4-methoxybenzylidene)-3-(2-hydroxy-3-(4-methylpiperazin-1-yl)propyl)imidazolidine-2,4-dione hydrochloride (5). (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (0.82 g) and 1-methylpiperazine (0.60 g) were irradiated at 600 W during 6 min and next at 750 W in 18 min (4 1 2 min, 2 1 3 min, 2 min, 2 1 1 min, 2 min). Methanol was used to crystallization. After conversion into the hydrochloride form crystallization with dry ethanol and next ethanol gave white crystals of 5 (0.18 g, 0.5 mmol, 17%), mp: 193–199 °C, R_f (III): 0.13. Anal. Calcd for C₁₉H₂₈N₄O₄Cl₂ 1 2.5 H₂O: C, 48.28; H, 6.16; N, 11.81. Found: C, 48.11; H, 6.59; N, 11.81. ¹H NMR (DMSO-d₆) d (ppm): 2.20–2.40 (m, 3H, N-CH₃), 2.72–3.12 (m, 8H, 2 1 Pp-2,3,5,6-H), 3.39–3.46 (m, 2H, N3-CH₂), 3.78 (s, 3H, OCH₃), 3.90–3.98 (br s, 1H, CHOH), 4.95–5.05 (br s, 1H, OH), 6.49 (s, 1H, CH@C), 6.95–9.98 (m, 2H, Ar-3,5-H), 7.59–7.62 (m, 2H, Ar-2,6-H), 10.65 (m, 1H, N1H).

4.1.2.2. (Z)-5-(4-methoxybenzylidene)-3-(2-hydroxy-3-(3-(4-methylpiperazin-1-yl)propylamino)propyl)imidazolidine-2,4-dione hydrochloride (6). (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (0.44 g) and 3-(4-methylpiperazin-1-yl)propan-1-amine (0.50 g) were irradiated at 600 W during 2 min, next at 750 W in 6 min (3 1 2 min) and next at 600 W in 8 min (4 1 2 min). Methanol was used to crystallization. After converting into the hydrochloride form, crystallizing with ethanol with water and precipitating with dry ethanol gave white crystals of 6 (0.15 g, 0.35 mmol, 22%), mp: 213–219 °C, R_f (III): 0.33. Anal. Calcd for C₂₂H₃₅N₅O₄Cl₂ 1 3H₂O: C, 47.10; H, 7.32; N, 12.52. Found: C, 47.31; H, 7.40; N, 12.54. ¹H NMR (DMSO-d₆) d (ppm): 1.95–2.05 (m, 2H, Pp-CH₂-CH₂), 2.47–2.50 (s, 3H, CH₃), 2.78–2.95 (m, 6H, Pp-CH₂, Pp-2,6-H), 2.98–3.15 (m, 4H, Pp-3,5-H), 3.20–3.30 (m, 4H, CH₂NHCH₂), 3.52–3.59 (m, 2H, N3-CH₂), 3.79 (s, 3H, OCH₃), 4.11 (m, 1H, CHOH), 5.95 (br s, 1H, OH), 6.51 (s, 1H, CH@C), 6.95–9.98 (d, J = 8.98 Hz, 2H, Ar-3,5-H), 7.60–7.63 (d, J = 8.98 Hz, 2H, Ar-2,6-H), 8.65–8.90 (m, 2H, NH₂⁺), 10.71 (s, 1H, N1H).

4.1.2.3. (Z)-5-(4-methoxybenzylidene)-3-(2-hydroxy-3-(piperidin-1-yl)propyl)imidazolidine-2,4-dione hydrochloride (7). (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (0.77 g) and 1-methylpiperazine (0.24 g) were irradiated at 600 W during 6 min and next at 750 W in 18 min (4 1 2 min, 2 1 3 min, 2 min, 2 1 1 min, 2 min). Methanol was used to crystallization. After conversion into the hydrochloride form crystallization with dry ethanol and next ethanol gave white crystals of 7 (0.04 g, 0.11 mmol, 4%), mp: 171–176 °C, R_f (III): 0.26. Anal. Calcd for C₁₉H₂₆N₃O₄Cl: C, 57.64; H, 6.62; N, 10.61; Found: C, 57.81; H, 6.34; N, 10.21. ¹H NMR (DMSO-d₆) d (ppm): 1.28–1.42 (m, 6H, Pip-3,4,5-H), 2.22–2.32 (m, 4H, Pip-2,6-H), 3.40–3.52 (m, 4H, N3-CH₂, Pip-CH₂), 3.78 (s, 3H, OCH₃), 3.91 (m, 1H, CHOH), 4.87–4.88 (m, 1H, OH), 6.43 (s, 1H, CH@C), 6.93–6.96 (m, 2H, Ar-3,5-H), 7.59–7.62 (m, 2H, Ar-2,6-H), 10.59 (s, 1H, N1H).

4.1.3. General procedure for preparation of amine derivatives of 5-arylideneimidazolidine-2,4-diones (8, 9)

(Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (2.2–5 mmol) and appropriate amine (2.2–5 mmol) were irradiated in a standard household microwave oven at various power (450–750 W) and time intervals (16–28 min) of irradiation for each prepared compound (8, 9), respectively. The reaction progress was controlled with TLC (V). After irradiation, the glassy residue was crystallized to give a pure product in basic form. The precipitate of pure product (8, 9) was separated by filtration.

4.1.3.1. (Z)-5-(4-methoxybenzylidene)-3-(3-(4-acetylpiperazin-1-yl)-2-hydroxypropyl)imidazolidine-2,4-dione (8). (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (1.37 g) and 1-acetylpiperazine (0.64 g) were irradiated at 450 W during 5 min (2 1 1 min, 3 min) next at 600 W during 4 min and next at 750 W during 7 min (2 min, 5 min). Crystallization with methanol with activated coal gave white crystals of 8 (1.21 g, 0.003 mmol, 60%), mp: 178–181 °C, R_f (III): 0.81. Anal. Calcd for C₂₀H₂₆N₄O₅: C, 59.69; H, 6.51; N, 13.92. Found: C, 59.63; H, 6.42; N, 13.90. ¹H NMR (DMSO-d₆) d (ppm): 1.93 (s, 3H, CH₃), 2.25–2.40 (m, 10H, Pip-2,3,4,5,6-H, Pp-CH₂), 3.46–3.49 (m, 2H, N3-CH₂), 3.78 (s, 3H, OCH₃), 3.95–3.96 (m, 1H, CHOH), 4.95–4.97 (d, J = 5.13 Hz, 1H, OH), 6.47 (s, 1H, CH@C), 6.94–6.97 (d, J = 8.97 Hz, 2H, Ar-3,5-H), 7.58–7.61 (d, J = 8.72 Hz 2H, Ar-2,6-H), 10.63 (s, 1H, N1H).

4.1.3.2. (Z)-5-(4-methoxybenzylidene)-3-(2-hydroxy-3-morpholinopropyl)imidazolidine-2,4-dione (9). (Z)-5-(4-methoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 33 (0.60 g) and morpholine (0.19 g) were irradiated at 450 W during 3 min, next at 600 W during 6 min (4 min, 2 min) and next 19 min (3 1 5 min, 4 min). Crystallization with methanol gave white crystals of 9 (0.18 g, 0.5 mmol, 23%), mp: 170–173 °C, R_f (III): 0.23. Anal. Calcd for C₁₈H₂₃N₃O₅: C, 59.82; H, 6.41; N, 11.63. Found: C, 59.85; H, 6.45; N, 11.49. ¹H NMR (DMSO-d₆) d (ppm): 2.27–2.32 (m, 4H, Mor-2,6-H), 2.34–2.39 (m, 2H, Mor-CH₂), 3.46–3.50 (m, 6H, CH₂OCH₂, N3-CH₂), 3.78 (s, 3H, OCH₃), 3.92–3.98 (q, J = 6.07 Hz, 1H, CHOH), 4.94–4.96 (d, J = 5.39 Hz, 1H, OH), 6.47 (s, 1H, CH@C), 6.94–6.97 (d, J = 8.72 Hz, 2H, Ar-3,5-H), 7.58–7.61 (d, J = 8.72 Hz 2H, Ar-2,6-H), 10.62 (s, 1H, N1H).

4.1.3.3. (Z)-5-(4-methoxybenzylidene)-3-(2-hydroxy-3-(piperazin-1-yl)propyl)imidazolidine-2,4-dione dihydrochloride (10). (Z)-5-(4-methoxybenzylidene)-3-(3-(4-acetylpiperazin-1-yl)-2-hydroxypropyl)imidazolidine-2,4-dione 8 (0.40 g) was added to 15% hydrochloric acid (2 mL). 10% NH_{3(aq)} was being added to hot solution until obtain pH 7. White crystals of 10 were

obtained (0.32 g, 8.9 mmol, 89%), mp: 269–272 °C, R_f (III): 0.23. Anal. Calcd for $C_{18}H_{26}N_4O_4Cl_2$: C, 59.99; H, 6.71; N, 15.55. Found: C, 59.61; H, 6.90; N, 15.19. 1H NMR (DMSO- d_6) δ (ppm): 1.21 (s, 1H, Pp-NH), 2.35–2.40 (t, def, 2H, Pp-CH₂), 2.57–2.61 (m, 4H, Pp-2,6-H), 2.99 (br s, 4H, Pp-3,5-H), 3.45–3.48 (d, J = 6.41 Hz, 2H, N3-CH₂), 3.78 (s, 3H, OCH₃), 3.92–3.94 (m, 1H, CHOH), 5.00–5.02 (d, J = 5.13 Hz, 1H, OH), 6.48 (s, 1H, CH@C), 6.94–6.97 (d, J = 8.72 Hz, 2H, Ar-3,5-H), 7.59–7.62 (d, J = 8.72 Hz, 2H, Ar-2,6-H), 7.68–9.25 (br s, 2H, Pp-NH⁺, Pp-NH₂), 9.5–11.2 (m, 1H, N1H).

4.1.4. General procedure for preparation of amine derivatives of 5-arylideneimidazolidine-2,4-diones (11–16)

5-Arylidene-3-(oxiran-2-ylmethyl)imidazolidine-2,4-diones 30, 31, 34 or 35 (10 mmol) and primary or secondary amine (40 mmol) and MeOH (30 mL) were mixed in flask. The mixture was refluxed for 3 h. The solvent was evaporated until dry residue was obtained. The residue was purified by crystallization with methanol.

4.1.4.1. (Z)-5-(4-chlorobenzylidene)-3-(2-hydroxy-3-(isopropylamino)propyl)imidazolidine-2,4-dione (11). (Z)-5-(4-chlorobenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 32 and isopropylamine were used to give white crystals of 11. 48%; mp: 184–186 °C, R_f (IV): 0.20. Anal. Calcd for $C_{16}H_{20}N_3O_3Cl$: C, 56.88; H, 5.97; N, 12.44. Found: C, 56.81; H, 5.68; N, 12.30. 1H NMR (DMSO) δ (ppm): 0.90 (d, J = 6.20 Hz, 6H, C-(CH₃)₂), 2.45–2.65 (m, 2H, CH₂NH), 2.72 (qu def., 1H, CH-(CH₃)₂), 3.40–3.55 (m, 2H, N3-CH₂), 3.75–4.00 (m, 1H, CHOH), 5.35 (s, 1H, OH), 6.50 (s, 1H, CH@C), 7.45 (d, J = 8.20 Hz, 2H, Ar-3,5-H), 7.70 (d, J = 8.00 Hz, 2H, Ar-2,6-H), 10.80 (s, 1H, N1H). IR KBr (cm^{-1}): 3435 (NH), 1770 (C4@O), 1725 (C2@O), 1580 (Ar).

4.1.4.2. (Z)-5-(4-chlorobenzylidene)-3-(2-hydroxy-3-(4-(2-hydroxyethyl)piperazin-1-yl)propyl)imidazolidine-2,4-dione (12). (Z)-5-(4-chlorobenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 32 and 2-(piperazin-1-yl)ethanol were used to give white crystals of 12. 82%, mp: 181–182 °C, R_f (IV): 0.26. Anal. Calcd for $C_{15}H_{25}N_4O_4Cl$: C, 55.80; H, 6.16; N, 13.16. Found: C, 55.52; H, 5.73; N, 13.16. 1H NMR (DMSO) δ (ppm): 2.20–2.40 (m, 10H, Pp-H, CH₂-CH₂-OH), 3.40–3.50 (m, 4H, CH₂-CHOH-CH₂), 3.78 (qu, J = 6.80 Hz, 1H, CHOH), 3.97 (t, J = 6.00 Hz, 2H, CH₂OH), 5.50 (s, 1H, OH), 6.50 (s, 1H, CH@C), 7.45 (d, J = 8.60 Hz, 2H, Ar-3,5-H), 7.65 (d, J = 8.50 Hz, 2H, Ar-2,6-H), 10.80 (s, 1H, N1H). IR KBr (cm^{-1}): 3410 (OH), 3310 (NH), 1760 (C4@O), 1720 (C2@O), 1590 (Ar).

4.1.4.3. (Z)-5-(4-bromobenzylidene)-3-(2-hydroxy-3-(isopropylamino)propyl)imidazolidine-2,4-dione (13). (Z)-5-(4-bromobenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 31 and isopropylamine were used to give white crystals of 13. 52%, mp: 180–182 °C, R_f (IV): 0.26. Anal. Calcd for $C_{16}H_{20}N_3O_3Br$: C, 50.26; H, 5.27; N, 10.92. Found: C, 49.86; H, 5.41; N, 10.43. 1H NMR for hydrochloride of 13 (DMSO) δ (ppm): 1.19 (d, J = 3.90 Hz, 3H, CH₃), 1.22 (d, J = 3.85 Hz, 3H, C-CH₃), 2.48 (qu, 1H, CH-(CH₃)₂), 3.04–3.10 (m, 1H, CHNH), 3.20–3.35 (m, 1H, CHNH), 3.40–3.55 (m, 2H, NCH₂), 4.09 (s, 1H, CHOH), 5.82 (d, J = 4.88 Hz, 1H, OH), 6.25 (s, 1H, CH@C), 7.43–7.45 (d, J = 6.70 Hz, 2H, Ar-2,6-H), 7.46–7.48 (d, 2H, J = 6.90 Hz, Ar-4,5-H), 8.40 (s, 1H, NH), 8.64 (s, 1H, NH⁺), 10.87 (s, 1H, N1H). IR KBr (cm^{-1}): 3440 (OH), 3440 (NH), 1765 (C4@O), 1715 (C2@O), 1595 (Ar).

4.1.4.4. (Z)-5-(4-(diethylamino)benzylidene)-3-(2-hydroxy-3-(4-(2-hydroxyethyl)piperazin-1-yl)propyl)imidazolidine-2,4-dione (14). (Z)-5-(4-(diethylamino)benzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 34 and 2-(piperazin-1-yl)ethanol were used to give white crystals of 14. 37%, mp: 108–110 °C, R_f (IV): 0.23. Anal. Calcd for $C_{23}H_{35}N_5O_4$: C, 61.90; H, 7.92; N, 15.72. Found:

C, 62.24; H, 7.72; N, 15.40. 1H NMR (DMSO) δ (ppm): 1.20 (t, J = 6.80 Hz, 6H, 2 \times CH₃), 2.20–2.50 (m, 12H, Pp-H, C₂H₄-OH), 3.30–3.60 (m, 8H, CH₂-CHOH-CH₂, CH₂NCH₂), 3.90–4.00 (m, 1H, CHOH), 4.30 (s, 1H, OH), 4.90 (1H, OH), 6.50 (s, 1H, CH@C), 6.60 (d, J = 8.80 Hz, 2H, Ar-3,5-H), 7.40 (d, J = 8.80 Hz, 2H, Ar-2,6-H), 10.30 (s, 1H, N1H). IR KBr (cm^{-1}): 3430 (OH), 3400 (NH), 1750 (C4@O), 1710 (C2@O), 1580 (Ar).

4.1.4.5. (Z)-5-(2,4-dimethoxybenzylidene)-3-(2-hydroxy-3-(isopropylamino)propyl)imidazolidine-2,4-dione (15). (Z)-5-(2,4-dimethoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 35 and isopropylamine were used to give white crystals of 15. 50%, mp: 148–150 °C, R_f (IV): 0.25. Anal. Calcd for $C_{18}H_{25}N_3O_5$: C, 59.50; H, 6.70; N, 10.94. Found: C, 59.32; H, 7.19; N, 11.53. 1H NMR for hydrochloride of 15 (DMSO) δ (ppm): 1.19 (d, J = 3.40 Hz, 3H, CH₃), 1.22 (d, J = 3.40 Hz, 3H, C-CH₃), 2.84 (qu def., 1H, CHNH), 3.00–3.15 (m, 1H, CHNH), 3.22–3.38 (m, 1H, CHNH), 3.40–3.58 (m, 2H, NCH₂), 3.80 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 4.10 (s, 1H, CH-OH), 5.80 (d, J = 6.46 Hz, 1H, OH), 6.50–6.69 (m, 2H, Ar-3,5-H), 6.71 (s, 1H, CH@C), 7.75 (d, 1H, J = 9.50 Hz, Ar-6-H), 8.35 (s, 1H, NH), 8.55 (s, 1H, NH⁺), 10.56 (s, 1H, N1H). IR KBr (cm^{-1}): 3420 (OH), 3380 (NH), 1750 (C4@O), 1720 (C2@O), 1580 (Ar).

4.1.4.6. (Z)-5-(2,4-dimethoxybenzylidene)-3-(2-hydroxy-3-(4-(2-hydroxyethyl)piperazin-1-yl)propyl)imidazolidine-2,4-dione (16). (Z)-5-(2,4-dimethoxybenzylidene)-3-(oxiran-2-ylmethyl)imidazolidine-2,4-dione 35 and 2-(piperazin-1-yl)ethanol were used to give white crystal of 16. 57%, mp: 162–163 °C, R_f (IV): 0.36. Anal. Calcd for $C_{21}H_{30}N_4O_6$: C, 58.05; H, 6.96; N, 12.89. Found: C, 58.10; H, 6.73; N, 12.84. 1H NMR (DMSO) δ (ppm): 2.20–2.40 (m, 12H, Pp-H, C₂H₄-OH), 3.40–3.50 (m, 4H, CH₂-CHOH-CH₂), 3.80 (s, 3H, OCH₃), 3.85 (s, 3H, OCH₃), 3.93 (m, 1H, CHOH), 4.95 (s, 1H, OH), 6.60 (d, J = 9.00 Hz, 2H, Ar-3,5-H), 6.70 (s, 1H, CH@C), 7.60 (d, J = 8.40 Hz, 1H, Ar-6-H), 9.60 (s, 1H, N1H). IR KBr (cm^{-1}): 3415 (OH), 3415 (NH), 1770 (C4@O), 1720 (C2@O), 1595 (Ar).

4.2. Molecular modeling methods

The 3D models of compounds 3–21 were built using Schrödinger Maestro³⁰ molecular modeling environment basing on the solved crystal structure of 19, crystallized in an unprotonated form and described previously.²⁴ Only (S)-isomers of alcohols 5–21 were taken into account. Compounds were submitted to further analysis as free bases. For each compound, a conformational search was then performed using the mixed torsional method as implemented in MacroModel 9.8 with MMFFs force field and TNCG (Truncated Newton Conjugate Gradient) method of energy minimization. The conformational analysis was carried out for aqueous solutions with continuum solvation treatment (Generalised Born/Solvent Accessible, GB/SA) and terminated when the root means square (RMS) of conjugate gradient was below 0.05 kJ mol⁻¹ Å⁻¹.

Found global minimum energy poses of the ligands were superimposed by a least-squares method; heavy atoms of hydantoin fragment (carbon, nitrogen and oxygen atoms) were chosen as fitting points (Fig. 3). The distances between structural fragments of molecules were measured in Maestro using centroids defined for a phenyl (AR) and a hydantoin ring (HC). For the graphic presentation of superimposed lowest energy conformations PyMOL software³¹ was used.

4.3. Microbiological assays

To determine minimal inhibitory concentrations (MICs), approximately 10⁶ cells were inoculated into 1 mL of Mueller-Hinton broth containing twofold serial dilutions of nalidixic acid

or chloramphenicol or sparfloxacin according to the Clinical and Laboratory Standards Institute (CLSI, <http://www.clsi.org/>) guidelines. Triplicate results were read after 18 h at 37 °C. To study synergistic activity, different fixed sub-inhibitory concentrations of the potential inhibitors, determined without antibiotic, were added during incubation with nalidixic acid or chloramphenicol or sparfloxacin. In addition, the reference efflux pump modulator, PAbN, was used at 0.050 mM. Control experiments were carried out without the different inhibitors. MIC values are means of three independent determinations.

Acknowledgements

Partly supported by grants: 501/N-COST/2009/0 (K/PMN/000031), COST action BM0701, Polonium 8127/2010 and ANR-11-BS07-019-02 'IBEF'.

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