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Inhibition of the Mex Pumps of *Pseudomonas aeruginosa* with a Newly Characterized Member of Peptidomimetic Family

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Authors' contributions

This work was carried out in collaboration between both authors. Author AA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author KR supervised the research and partially financed the study and was involved in all parts of the work. Both authors have read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Efflux pumps, particularly resistance-nodulation-division family such as Mex pumps in *Pseudomonas aeruginosa,* are the major contributors to multidrug resistance in Gram-negative bacteria. Since the well-known efflux pump inhibitor (EPI), phenylalanine-arginine β-naphthylamide (PAβN), is a substrate of cathepsin C (a proteolytic enzyme), inhibitory effects of similar substrates of this enzyme, proline-arginine 4-methoxy-β-naphthylamide (PA4MβN) and N_α-Benzoyl-DL-arginine β-naphthylamide (BAN), were investigated as possible new EPIs. Additionally, the probable EPI activities of three different alkaloids (noscapine, caffeine and ergotamine) against Mex-pump expressing strains were also evaluated mainly based on their structural similarities to major EPIs.
Methodology: MPC_8 (minimum potentiating concentrations) of mentioned compounds in combination with levofloxacin (LVX) were screened against a wild type (PAO1) and two different Mex-pump overexpressing *P. aeruginosa* strains (*nalB* and *nfxB*). The most synergizing compound was further validated by the checkerboard assay for its EPI potency against strains overexpressing

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or lacking different Mex-pumps. The frequencies of emergence of LVX-resistance strains when it was used either alone or in combination with the suggested EPI were also compared against each other.

Results: PA4MβN caused significant increase in LVX activity against PAO1 and different Mex-pump overexpressing *P. aeruginosa* strains showing a significant decrease in the intrinsic (8-fold) and acquired (16-fold) resistances. Interestingly, PA4MβN fully prevented further emergence of *P. aeruginosa* mutants highly resistant to fluoroquinolones.

Conclusion: A new compound from peptidomimetic family (PA4MβN), which is also a substrate of cathepsin C, was introduced in the current study as an effective EPI against Mex pumps from *P. aeruginosa*.

Keywords: Cathepsin C; efflux pump inhibitor; multidrug resistance; Mex pump; proline-arginine 4-methoxy-β-naphthylamide; N_α-Benzoyl-DL-arginine β-naphthylamide.

1. INTRODUCTION

Multidrug resistance (MDR) is recognized as a major threat to human health [1]. *Pseudomonas aeruginosa*, the archetype of MDR phenotype, causes serious life-threatening infections with a high mortality rate mostly because of its innate resistance to numerous antibiotics and its ability to acquire high-level MDR to nearly all available antimicrobial drugs [2]. Of the various enzymatic and mutational resistance mechanisms, multidrug efflux pumps belonging to the resistance-nodulation-division (RND) family of drug/proton antiporters appear to be the most significant contributors to clinically relevant MDR in *P. aeruginosa* [2,3]. RND pumps act as the first line of bacterial defense that reinforce the development of additional resistance mechanisms allowing the emergence of multidrug and even pandrug-resistant (PDR) bacteria [4].

Efflux pumps have been reported to capture various antimicrobial agents including antibiotics and expel them into the outside media preventing their accumulation at the toxic level within the bacterial cells and therefore allowing them to survive at higher antibiotic concentrations [5]. In *P. aeruginosa*, RND pumps that are reported for their prevalent expressions in clinical isolates include MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM [2,6,7,8]. Even though antimicrobial efflux is not the only mechanism of resistance to a particular agent, numerous studies have demonstrated that deletion of such efflux pumps, especially MexAB-OprM, renders the bacterial strains susceptible to many drugs [2,3,9,10]. Fluoroquinolones (FQs), which are the preferred substrates for almost all Mex pumps, frequently are coupled with emergence of high MDR and even PDR isolates via triggering the overexpression of RND efflux systems and subsequent cross resistance to nearly all other substrates of these pumps [7].

Given the clear involvement of the efflux pumps in the development of MDR-related clinical isolates especially among Gram-negative bacteria and due to the scarcity of new antibiotics to combat these resistant pathogens, identification of efficient inhibitors being able to circumvent or block antibiotic efflux activities is a promising strategy to provide effective treatments of MDR-infections [11]. Efflux pump inhibitors (EPIs) can be used in combination with antibiotics to restore the maximum capacities of antimicrobials [11-14].

Phenylalanine-arginine β-naphthylamide (PAβN) (also known as MC-207,110) (Fig. 1) is one of the most studied and most developed EPIs of Mex pumps [11,15,16]. PAβN and its two major derivatives (MC-02,595 and MC-04,124) (Fig. 1) belong to the peptidomimetic family (also known as C-capped dipeptides) [16].

PAβN has shown a broad spectrum EPI activity against all four clinically relevant *P. aeruginosa* Mex pumps and similar RND pumps from various Gram-negative bacteria and even against major facilitator superfamily efflux pumps in some Gram-positive bacteria [17-19]. PAβN could expand the spectrum of antibacterial activity of levofloxacin (LVX; the second generation of FQs), reduce the level of intrinsic resistance of *P. aeruginosa* to LVX and revert its acquired resistance arisen from the efflux pump overexpression to the antibiotic. More importantly, PAβN could decrease the emergence frequency of highly resistant mutants of *P. aeruginosa*. PAβN can also boost the activities of other antibiotic substrates of Mex pumps but cannot potentiate the activities of the antibiotics other than their specific substrates of such pumps [11,15].

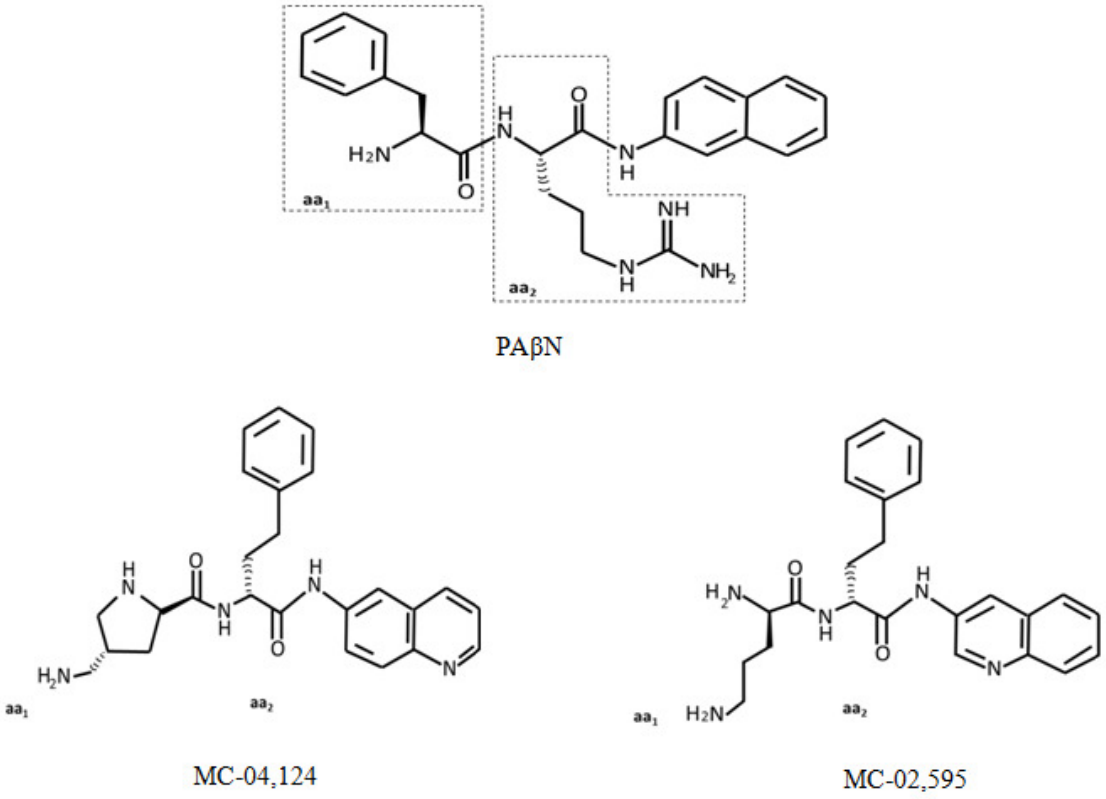

Fig. 1. Chemical structures (drawn by PowerPoint™, Office 2013 package, Microsoft Inc., Redmond, Washington DC) of PAβN and its two derivatives [11,13,14,16]

aa: amino acid.
PAβN: Phenylalanine-arginine β-naphthylamide,
MC-02,595: D-ornithine-D-homophenylalanine-3-aminoquinoline,
MC-04,124: L-proline-D-homophenylalanine-3-aminoquinoline

In view of the remarkable EPI activity of PAβN against RND efflux pumps [15], the attention in the current study was focused on the discovery of novel potential EPIs. Therefore, on the basis of specific chemical structure of PAβN, two other compounds [proline-arginine 4-methoxy-β-naphthylamide (PA4MβN) and N_{α}-benzoyl-DL-arginine β-naphthylamide (BAN)] (Fig. 2) are being considered as potential EPIs in the current study. The former compound is a substrate of dipeptidyl peptidase I (DPPI, also known as cathepsin C) and the latter is a substrate for some related proteolytic enzymes such as cathepsin H and cathepsin B1 [20,21]. Considering such functional and structural similarities between PAβN and the two introduced compounds, the EPI potential of the compounds was investigated in this study.

The presence of heterocyclic structure with one or two nitrogen atom(s) in the ring moiety(s) is prevalent in almost all effective EPI compounds [22,23]. Also, numerous in-vitro studies provide strong evidences that some phytochemicals and microbial products such as alkaloids can exhibit EPI activities against *P. aeruginosa* Mex pumps and their homologues in various Gram-negative bacteria [11,13,24,25]. On that aspect, three alkaloids (noscapine, caffeine and ergotamine) (Fig. 3) containing *iso*-quinoline (in noscapine), purine (in caffeine) and oxazole, pyrrole, pyrazine, indole and quinoline (in ergotamine) were selected as potential EPIs against Mex pumps. Therefore, the objectives of the current study were (i) to evaluate the overall activities of PA4MβN, BAN, noscapine, caffeine and ergotamine on *P. aeruginosa* isolates expressing different Mex pumps and (ii) to assess their abilities in combination with LVX to restore antibiotic susceptibility of MDR *P. aeruginosa* by inhibiting the activities of Mex pumps.

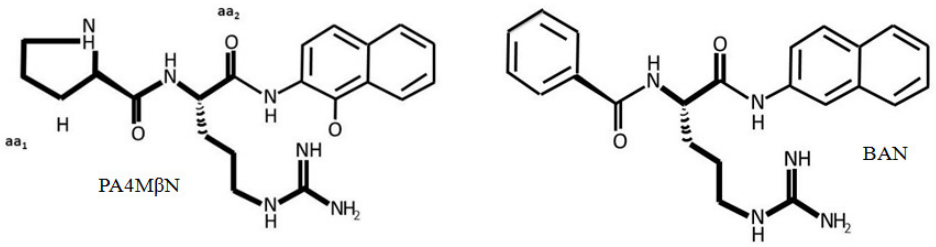

Fig. 2. Chemical structures of PA4MβN and BAN drawn by PowerPoint™ (Office 2013 package, Microsoft Inc., Redmond, Washington DC)
PA4MβN: Proline-arginine 4-methoxy-β-naphthylamide,
BAN: N_{α}-Benzoyl-DL-arginine β-naphthylamide

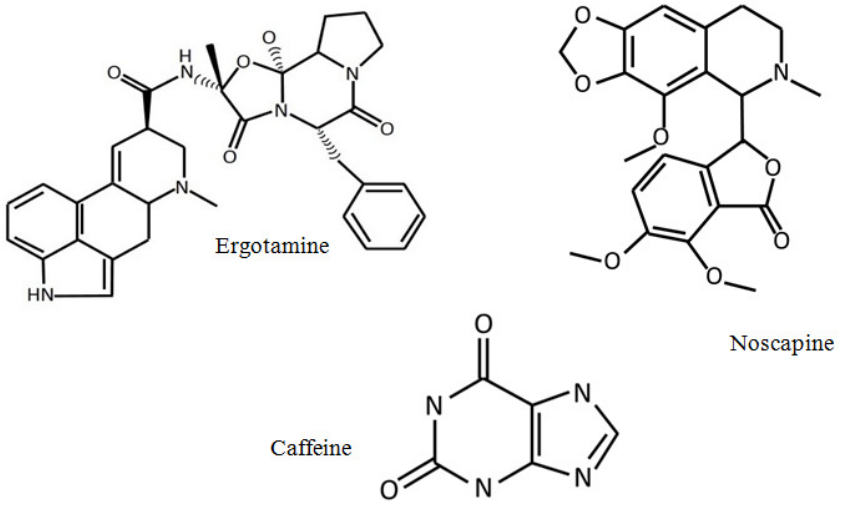

Fig. 3. Chemical structures of studied alkaloids drawn by PowerPoint™ (Office 2013 package, Microsoft Inc., Redmond, Washington DC)

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media

All *P. aeruginosa* strains used in this study (Table 1) were kindly provided by Dr. Keith Poole (Queen's University, Kingston, ON) except for *P. aeruginosa* ATCC 27853, which was used as the control strain to verify the accuracy of susceptibility test results. Cation-adjusted Mueller–Hinton broth (MHB) and Mueller-Hinton Agar (MHA) (Merck Chemical Company, Darmstadt, Germany) were used to perform antibacterial susceptibility tests.

2.2 Chemical Compounds

PA4MβN and BAN, caffeine, noscapine hydrochloride, gentamicin sulfate, dimethyl sulphoxide (DMSO), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Aldrich (St. Louis, MO). Ergotamine D-tartrate was from Fluka Chemie AG (Buchs, Switzerland) and levofloxacin (LVX) was obtained from Serva Feinbiochemica (Heidelberg, NY).

2.3 Determination of Minimum Inhibitory Concentrations (MICs) of Five Candidate Compounds

For all the candidate compounds (caffeine, noscapine, ergotamine, BAN and PA4MβN), MICs were determined over the concentration range of 1-1000 µg/ml (using a two-fold dilution series) against PAO1 and *nalB P. aeruginosa* strains in 96-well microtiter plates using the broth microdilution technique according to the

guidelines of the Clinical and Laboratory Standards Institute (CLSI) [26]. MHB containing two-fold serial dilutions of each compound (at twice the required concentrations) was dispensed (100 µl) into the wells of the microplates.

Table 1. List of different strains from *P. aeruginosa* used in the current study.

Strain	Relevant characteristics
K767	PAO1 (Wild type)
K1455	K767 *nalB* mutant (Overexpressing MexAB-OprM)
K1536	K767 *nfxB* mutant (Overexpressing MexCD,OprJ)
K1523	K767 Δ*mexB*
K1542	K767 Δ*mexXY*, Δ*mexB*
K2896	K767 Δ*mexCD-oprJ*, Δ*mexXY*, Δ*mexB*
K2153	PA2 (clinical isolate)
ATCC 27853[a]	*P. aeruginosa* reference strain

[a] *ATCC, American Type Culture Collection*

The bacterial suspensions containing approximately 1.5×10^8 CFU/ mL (determined by OD_{620} of 0.08-0.1) in MHB were prepared and 10^5 CFU/well were added to a final volume of 200 µl.

Plates were incubated at 37°C for about 22 h. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited the growth of tested isolates as observed with the unaided eyes [26]. All candidate compounds were dissolved in distilled water except for noscapine and BAN, which were dissolved in DMSO and diluted in MHB at sub-inhibitory concentrations (<4%, v/v), in order to avoid solvent's direct antibacterial effect [27]. Three replicates of each microassay were carried out and the experiment was carried out three times to verify the accuracy of the results.

2.4 Evaluation of the Synergy Levels between Levofloxacin and the Candidate Compounds

All five candidate compounds were further screened for their synergistic effects with LVX as a marker of Mex pumps activities (0.008-8 µg/ml) against PAO1 and *nalB P. aeruginosa* strains using the standard microdilution method [9,26]. Noscapine, caffeine and ergotamine (alkaloids) were tested at seven concentration levels (20, 40, 50, 100, 200, 500, 1000 µg/ml). BAN and PA4MβN were tested at four concentrations (20, 40, 50, 100 µg/ml) [28]. Each assay was carried out in triplicates.

To quantify the potency of compounds as EPIs, a new parameter (MPC_8) was also defined as the minimum concentration of an EPI required (<40 µg/ml) to decrease the MIC of the combination agent (e.g., antibiotics) 8-fold [29,30]. The compound(s) causing eight-fold or greater reduction in the MIC of LVX were further tested by checkerboard assay [15,28,31], which was used for a more detailed study of interactions between LVX and the most potent EPI candidate compound(s) against the studied strains of *P. aeruginosa* lacking or overexpressing efflux pumps (Table 1). LVX was tested at 0.008-8 µg/ml, whereas potential EPIs were tested at concentrations within 0.06 to 40 µg/ml according to previous results obtained from the assay with fixed concentrations of EPI candidates. MIC of LVX alone was investigated in the first row and combined with the EPIs in the remaining rows. The MIC of gentamicin (as a negative reference) at all concentrations of LVX in this study in combination with the most potent EPI candidate compound (at 0.06-40 µg/ml) was also examined against *nalB* mutant.

2.5 Comparison of the Frequency of Levofloxacin-resistant Strains Emergence with or Without the Potential EPI(s)

Strains of *P. aeruginosa* with MIC levels equal to or higher than 2 µg/ml are considered to be clinically resistant to antimicrobial agents [15,29]. Therefore, experiments were performed with 1.0 µg/ml of LVX (i.e., four times the MIC level obtained for PAO1 strain in the current study, 0.25 µg/mL). *P. aeruginosa* PAO1 at 100 µg/ml concentration was plated onto MHA containing LVX alone or LVX in combination with the potential EPI at two concentration levels of 20 and 40 µg/ml (based on MPC_8 definition) [28]. The frequency of resistance was determined as the ratio of number of bacteria (CFU per ml) that appeared after overnight incubation on LVX-containing MHA plates (with/without EPI) to the number that appeared after overnight incubation on LVX-free MHA plates [29].

2.6 *In silico* Investigation of Probable Homology between Amino Acid Sequences of RND Components of Mex Systems and that of Cathepsin C

Similarity in the amino acid sequences of RND subunits of Mex systems (MexB, MexD, MexF and MexY) and those of cathepsin C was the hypothesis for potential EPI activity of PA4MβN. Therefore, amino acid sequence alignments of *P. aeruginosa* Mex pumps were performed with both human pre-pro-cathepsin C and mature cathepsin C sequences. Protein alignments were obtained by using Basic Local Alignment Search Tool for Proteins (BLASTP) from the National Center for Biotechnology Information (Bethesda, MD) [32].

3. RESULTS

3.1 MIC-based Synergy Evaluation in the Presence of Candidate Compounds

To make sure no direct (intrinsic) antibacterial effects are involved in the EPI activities of alkaloids, BAN and PA4MβN, the MICs of all of these compounds were separately determined against PAO1 and *nalB* strains. All the compounds alone were devoid of any direct antibacterial activities (MIC > 400 µg/ml) [28] (Table 2). The MIC levels of LVX in combination with the candidate compounds against *P. aeruginosa* PAO1 and *nalB P. aeruginosa* strains were also obtained (Table 2). The combination that showed significant synergy was that of PA4MβN with LVX. For *P. aeruginosa* PAO1, the MIC of LVX in the presence of PA4MβN (50 and 100 µg/ml) decreased 32-fold (Table 2). The reduction was 8-fold in the presence of PA4MβN (20 and 40 µg/ml) (Fig. 4).

For *P. aeruginosa nalB* mutant, the MIC levels for LVX in the presence of PA4MβN at 50 and 100 µg/ml were decreased to the same level of 256-fold. But, it was decreased to the levels 8- and 16-fold, respectively (Table 2 & Fig. 4), when using PA4MβN at 20 µg/ml and 40 µg/ml concentrations.

The combination of LVX and BAN (at 40, 50, 100 µg/ml) displayed only 2-fold reduction against PAO1, *nalB* and *nfxB* strains and no reduction when LVX was used with 20 µg/ml of BAN. No synergy was found for all the three alkaloids (20, 40, 50, 100, 200, 500, 1000 µg/ml) with LVX against all the above-mentioned strains (Table 2).

Only PA4MβN was the most significant LVX-potentiator against Mex pump-expressing resistant strains of *P. aeruginosa* (highlighted box in Table 2). Therefore, this compound was selected for further study. The three alkaloids and BAN were excluded from further analysis for their potential EPI activities since their MPC_8 values were above 40 µg/ml.

3.2 EPI Activity of the Most Potent Candidate Compound, PA4MβN

Potential EPI activities of PA4MβN were determined by evaluating the interactions between LVX and PA4MβN against strains both overexpressing and lacking different Mex pumps in *P. aeruginosa* (Figs. 4 & 5). When LVX was used in combination with PA4MβN, its MIC against PAO1 strain was reduced from 0.25 to 0.03 µg/ml (up to 8-fold) (Fig. 4).

Also, PA4MβN caused significant (16-fold) decrease in MICs of LVX against both *nalB* (from 2 to 0.125 µg/ml) and *nfxB* (from 4 to 0.25 µg/ml). PA4MβN rendered the clinical isolate (PA2) more susceptible (8-fold) to LVX (from 1 to 0.125 µg/ml) (Fig. 4). MPC_8 values of PA4MβN for all efflux-pump-expressing strains were less than 40 µg/ml and showed a good synergy with LVX (Table 3).

PA4MβN showed 4-fold reduction in the MIC of LVX against *ΔmexXY, ΔmexB*, and *ΔmexXY, ΔmexB, ΔmexCD-oprJ* mutants and 8-fold decrease against *ΔmexB* mutant (Fig. 5). To verify the reduction levels of LVX MICs via PA4MβN against these susceptible strains, we used CCCP instead of PA4MβN. Similar to other RND pumps, Mex pumps in *P. aeruginosa* utilize proton motive force to efflux the drugs and CCCP (as an ionophore proton conductor) could destroy the proton gradient across bacterial inner membrane and inhibit Mex pumps indirectly [33]. MIC levels obtained for LVX in combination with CCCP against strains lacking efflux pumps were in the exact ranges as those of PA4MβNs.

When used with gentamicin (as negative reference), PA4MβN displayed no reduction in the MIC of this antibiotic against *nalB* mutant.

3.3 Emergence of Highly Levofloxacin-Resistant Strains in the Presence of PA4MβN

Investigating the frequency of emergence of resistant mutant (MIC > 1 µg/ml; four times the MIC for PAO1 strain) of *P. aeruginosa* PAO1 in the presence of PA4MβN showed that in the case of LVX alone, the frequency of such emergence was ~10^{-9} CFU/mL and surprisingly no bacterial growth was observed on the plates containing LVX and EPI combined.

3.4 Homology between Amino Acid Sequences of Mex Systems and Cathepsin C

Despite all the extensive efforts to improve EPI activities of PAβN and its derivatives, according to the authors' knowledge, the primary function of PAβN was never mentioned before its discovery as an EPI. PAβN is a chromogenic substrate of cathepsin C, which is an abundant lysosomal cysteine protease that sequentially removes dipeptides from the free N termini of proteins and peptic substrates [20].

As is the case for PAβN, PA4MβN is also a chromogenic substrate for cathepsin C. BAN is also a chromogenic substrate for such proteolytic enzymes as cathepsin B1 and cathepsin H [21]. X-ray co-crystallization structure studies of AcrB (highly close homologue of MexB) [34,35] and cathepsin C [20,36] combined with their specific ligands (such as ciprofloxacin for AcrB and Gly-Phe-diazomethane for cathepsin C) have revealed their possible substrate binding sites and have also showed the exact residues engaged in trapping the substrates.

Amino acid sequence alignments of *P. aeruginosa* Mex pumps (with human pre-pro-cathepsin C or mature cathepsin C sequences in the current study) revealed no considerable similarities especially among the residues predicted to be involved in binding to the substrates of both proteins. Furthermore, two separated alignments of the amino- (from Met1 to Arg529) and the carboxyl-termini (Gly530 to Gln1046) halves of MexB with cathepcin c sequence also showed no apparent similarity. Consequently, this hypothesis was nullified.

Table 2. MIC level (µg/ml) of levofloxacin (LVX) alone and those in combinations with the candidate compounds against PAO1 and *nalB* strains

		MIC (µg/ml)	
	EPIs	**PAO1**	***nalB***
LVX	**PA4MβN[a]**		
+	-	0.25	2
+	+ [**]	**0.008 (32)**[*]	**0.008 (256)**[*]
-	+	> 500	> 500
LVX	**BAN[b]**		
+	-	0.25	2
+	+	0.125 (2)[*]	1(2)[*]
-	+	400 > MIC <450	400 > MIC < 450
LVX	**Noscapine[c]**		
+	-	0.25	2
+	+	0.25	2
-	+	> 1000	> 1000
LVX	**Ergotamine[c]**		
+	-	0.25	2
+	+	0.25	2
-	+	> 10000	> 10000
LVX	**Caffeine[c]**		
+	-	0.25	2
+	+	0.25	2
-	+	> 1000	> 1000

[a] PA4MβN at 50, 100 µg/ml; [b] BAN at 40, 50, 100 µg/ml; [c] Noscapine, ergotamine and caffeine at 20, 40, 50, 100, 200, 500, 1000 µg/ml, [*] Numbers in the brackets indicate the fold reduction of levofloxacin (LVX) MIC in the presence of potential inhibitors, [**] The highlighted combination (LVX+ PA4MβN) was selected for the next stage of current study (due to the significant low LVX levels)

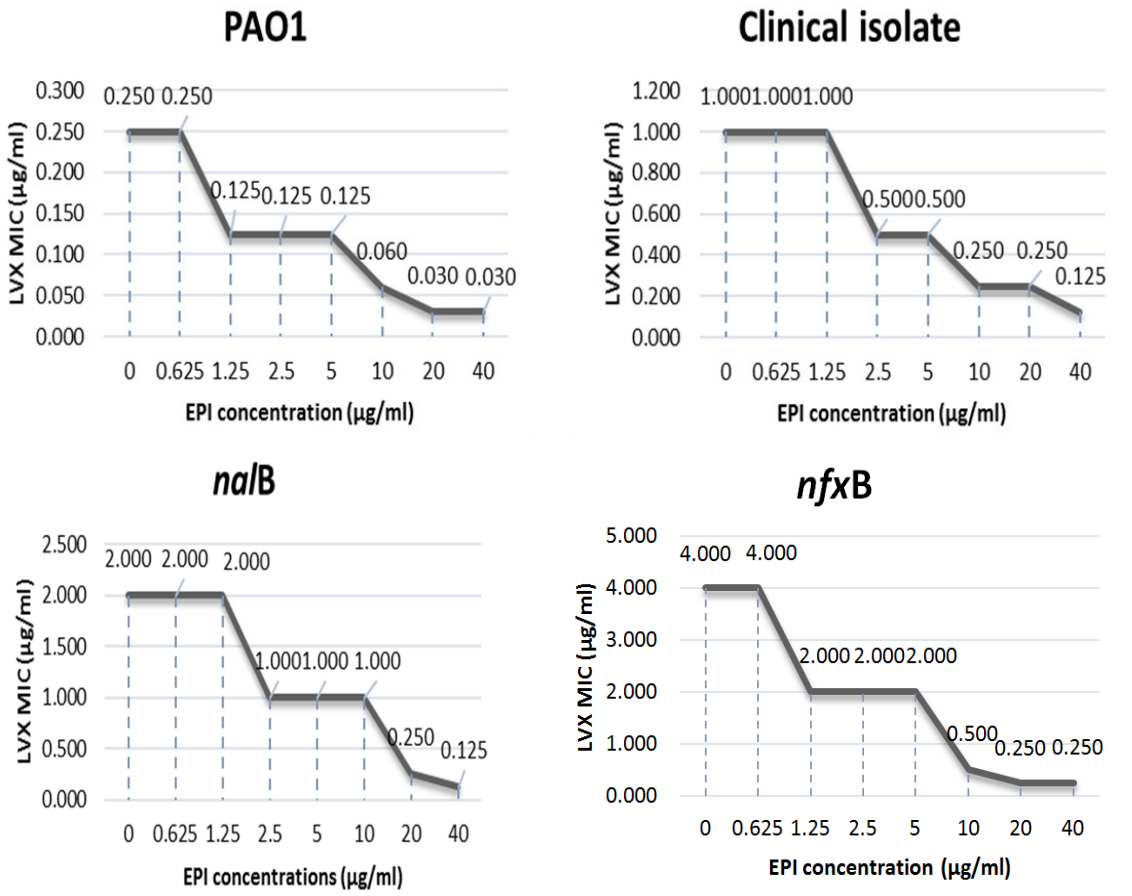

Fig. 4. MICs (µg/ml) of levofloxacin (LVX) in combination with different concentrations (µg/ml) of PA4MβN against wild type and some strains of *P. aeruginosa* overexpressing different Mex pumps

Relevant characteristics of all of the strains are listed in Table 1.
EPI: PA4MβN

Table 3. MPC_8 (µg/ml) and EC_{50} (µg/ml) of PA4MβN on various Mex pumps expressing *P. aeruginosa* (based on Fig. 4 & Fig. 5)

Strain	Genotype	MPC_8[a]	EC_{50}[b]	E_{max}[c]
K767	wild type	20	10	8
K2153	clinical isolate	40	10	8
K1455	*nalB*	20	20	16
K1536	*nfxB*	10	10	16
K1523	Δ*mexB*	40	20	8
K1542	Δ*mexXY*,Δ*mexB*	ND[d]	15	4
K2896	Δ*mexXY*,Δ*mexB*, Δ*mexCD-OprJ*	ND	10	4

[a] *MPC_8: minimum concentration (µg/ml) of PA4MβN required to decrease LVX MIC 8-fold.*
[b] *EC_{50}: concentration (µg/ml) of PA4MβN at which half of the LVX potential effect is achieved.*
[c] *E_{max}: ratio between the LVX MIC without PA4MβN and with the presence of a maximal potentiating concentration of PA4MβN,* [d] *Not detected*

4. DISCUSSION

Among all EPI candidates, PA4MβN with significant structural resemblance to PAβN displayed potential EPI activity against Mex pumps. According to the results of our study, PA4MβN satisfied five major criteria as follows for an effective EPI:

(i) PA4MβN did not indicate an intrinsic antimicrobial activity against *P. aeruginosa* strains (Table 2). Logically, an effective EPI should not possess any intrinsic antibacterial activity, as active efflux pumps are not necessary for viability of the bacteria [28].

(ii) PA4MβN resulted in significant increase in the activity of LVX, known substrate for Mex pumps, against different Mex pump-expressing strains. Under natural conditions, PAO1 expresses MexAB-OprM constitutively [37], so PA4MβN significantly (8-fold) decreased the intrinsic resistance of *P. aeruginosa*. Also, PA4MβN was capable of defeating acquired resistance (16-fold) due to the overexpression of MexAB-OprM and MexCD-OprJ in *nalB* and *nfxB* mutants, respectively (Fig. 4).

(iii) Gentamicin (similar to other aminoglycoside antibiotics) is not a substrate of MexAB-OprM [15]. Therefore, PA4MβN (at all the studied concentration levels) showed no reduction in MIC of gentamicin against *nalB* mutant. However, at 40 µg/ml, PA4MβN caused 2-fold increase in MIC of gentamicin (2 µg/ml against *nalB* if used alone). *mexXY* is expressed at sufficient level in *nalB* and it could be up-regulated in the presence of aminoglycosides [38,39]. Therefore, together with inhibitory effect of PA4MβN against MexAB-OprM, overexpressed MexXY-OprM achieves the additional energy (proton ions) to efflux extra amount of gentamicin molecules and as a result causes an increase in the MIC of the antibiotic.

(iv) A major expectation from an EPI is that it should not significantly reduce the activity of the antibiotics against strains lacking efflux pumps. Therefore, PA4MβN did not result in significant increase in the antibiotic activity against strains lacking efflux system(s) (Fig. 5).

(v) More importantly, PA4MβN fully prevented further emergence of *P. aeruginosa* mutants highly resistant to FQs, the key antibiotic class in MDR development.

It has been demonstrated [15,29] that deletion or inhibition of efflux pumps in *P. aeruginosa* significantly decreases the frequency of emergence of highly resistant strains. Therefore, PA4MβN could completely prevent the expression of other resistance mechanisms and eventually emergence of highly FQ-resistant strains. Such finding confirmed the prominent involvement of Mex pumps in the emergence of highly FQs resistant strains as the inhibition of these pumps via PA4MβN did not allow the emergence of any LVX-resistant strains from *P. aeruginosa* used in the current study.

The correlation between the presence of the pumps and the degree to which LVX activity was potentiated by PA4MβN is suggesting a competitive EPI activity. Also, for both of the over-expressed strains (*nalB* and *nfxB*), the degrees of potentiation by PA4MβN were higher than those in the strains lacking Mex pump(s). Therefore, the potentiation activity of the compound was efflux-pump-dependent. Consequently, from a microbiological point of view, PA4MβN can meet the expectations of this study as an effective EPI.

Structurally, PAβN, PA4MβN and BANA contain β-naphtylamide and arginine moieties in their capping moiety and middle amino acid section, respectively (Fig. 1 & Fig. 2). The major difference between PA4MβN and PAβN is the presence of *L*-proline instead of *L*-phenylalanine in the first amino acid position. On the other hand, compared to PAβN, BAN has a benzoyl group instead of *L*-phenylalanine in its chemical structure (Fig. 2) and this feature probably is the main reason for no EPI activity of BAN. Therefore, amino acids *L*-phenylalanine from PAβN and *L*-proline from PA4MβN play major roles in their EPI activities. Comparing PA4MβN with PAβN, it can be suggested that the presence of two amino acids preferably one positively charged aliphatic and other a heterocyclic compound containing nitrogen in the overall structure of a C-capped dipeptide is a prerequisite for an EPI activity.

To optimize the biological, physicochemical and pharmacological properties of PAβN, two derivatives of this compound (namely MC-02,595 and MC-04,124) (Fig. 1) were introduced [30,40,41]. PAβN and the two mentioned peptidomimetic derivatives became leaders of RND EPIs that are also known as C-capped dipeptides [40]. Comparing the structures of a large number of successful PAβN analogues synthesized during the optimization stages of PAβN EPI properties shows that the existence of certain type of peptide moieties is essential for EPI activity of a compound.

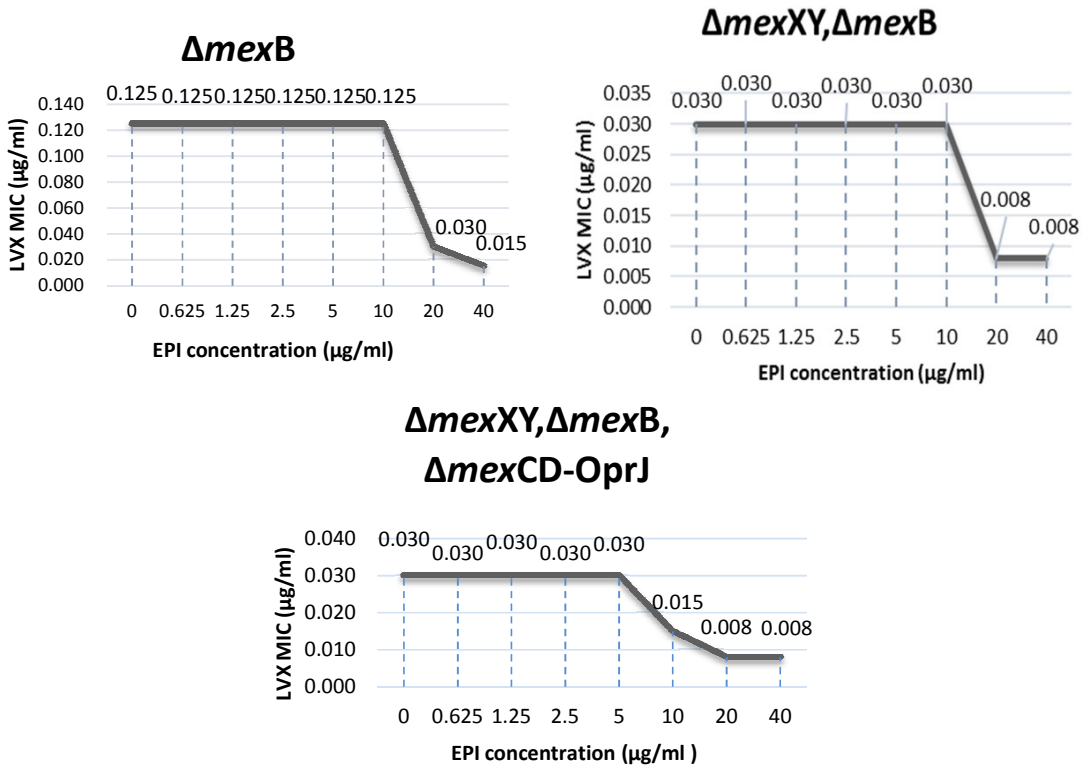

Fig. 5. MICs (µg/ml) of levofloxacin (LVX) in combination with different concentrations (µg/ml) of PA4MβN against some strains of *P. aeruginosa* lacking different Mex pumps

Similar to the other RND transporters, Mex pumps are organized as asymmetric trimers where each protomer consists of 12 transmembrane (TM) helices and an extensive periplasmic domain formed by two loops between TM1 and TM2, and between TM7 and TM8 [42]. In *P. aeruginosa*, Asp407 and Asp408 in TM4 and also Lys939 in TM10 appear to constitute a salt-bridged network [42] stabilized by hydrogen bonding of Thr976 in TM11 [42]. During the contact of efflux pumps with the drugs, a proton binds with Asp407 and disturbs the salt-bridge/H-bond interactions resulting in proton influx into the cytoplasm and eventually efflux of the drug molecules [42]. TM4 is the major route for the proton translocation [42]. Three potential drug-binding sites in AcrB (a close homologue to MexB) include an extremely large central cavity [34], a deep external depression formed by the C-terminal periplasmic loop domain on the outside surface of this domain [35], and a deep inside periplasmic domain [42]. In the first step of substrate recognition, different ligands bind to the various positions of the central cavity, which is formed by the TM domains of the three protomers of RND pumps on the interface between periplasm and the inner membrane [34]. According to a co-crystallographic study of AcrB with four structurally diverse ligands, all initial substrate bindings occur mainly in the periplasmic loop regions between TM3 and TM4 loops and also between TM5 and TM6 loops within the wall of the upper portion of central cavity [34]. Four well-conserved phenylalanine residues (Phe386, Phe388, Phe458 and Phe459) in these regions (TM5 and TM6) play a key role in primary substrate interactions with RND pumps [34]. In a study on AcrBN109A mutant bound to similar agents to the those mentioned above plus PAβN, it was found that most ligands are attached very close to the top of TM5 and TM6; while PAβN was attached to the location somewhat to the left of the others; that is, towards the TM3 and TM4 [35].

In the current study, it was assumed that PAβN and PA4MβN not only binds with the usual residues responsible for such connection, but also interact with Phe396 inside TM4.

Given the apparent importance of TM4 in proton influx, most probably, this unusual interaction adversely disturbs the regular hydrogen-bonding interactions of the backbone NH and C=O groups characteristic of the α-helix from TM4. Accordingly, uncommon involvement of TM4 in substrate binding result in the unexpected separation of Asp407 and Asp408 from the other two members of the salt bridge/H-bond network (Lys939 and Thr978) in a way that the network can no longer be protonated and therefore supply of energy to RND pumps is disturbed. Since the drug efflux is coupled to the proton influx process [43], unless a proton translocation takes place, drug efflux to the external medium would not occur and as a consequence the pump function would be inhibited. Furthermore, binding of the drugs triggers a number of consecutive conformational changes in RND pumps that ultimately result in drug efflux [43,44]. These changes start with proton acquisition by a proton relay network. Therefore, PAβN causes (via disturbing the regular α-helix structure in TM4) an indirect disruption of the network and prevents the subsequent conformational changes that are needed for the proper binding of the substrates. It seems that the carboxylate oxygen of Phe396 binds to the imino group of proline in PA4MβN or amino group of phenylalanine in PAβN. Therefore, these two specific amino acids located in the first section of PA4MβN and PAβN structures play an important role with regard to their EPI activities. Absence of amino group in the first part of the structure in BAN and subsequent failure to establish hydrogen bond with Phe396 residue in TM4 is possibly the reason for the compound not being able to inhibit the Mex pumps activity.

5. CONCLUSION

In the current study we describe the preliminary *in vitro* characterization of PA4MβN, a new member of peptidomimetics family with inhibitory effect on the Mex pumps of *P. aeruginosa*. More research is needed on several other peptidomimetic compounds or substrates of cathepsin C to verify such findings as what obtained in the current study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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