Original Article

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Microbiology Section

Possible Role of Curcumin as an Efflux Pump Inhibitor in Multi Drug Resistant Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: Multidrug resistant non-fermenters are continuously increasing in hospital and ICU settings. One of the mechanisms of developing drug resistance is possession of efflux pump through which bacteria extrude antimicrobial agents and other toxic substances. If these efflux channels are blocked or inhibited, increased drug concentration can be achieved in a bacterial cell with optimal drug dose. Present study was aimed to investigate role of curcumin as efflux pump inhibitor (EPI) and to compare its activity with a known EPI like phe-arg-beta-naphthylamide (PAβN).

Materials and Methods: A total of 170 clinical isolates of *Pseudomonas aeruginosa* were taken, antimicrobial susceptibility was performed by disc diffusion test and minimum inhibitory concentration (MIC) against selected drugs before and after adding known synthetic EPI, PAβN (20mg/L). Out of these, 30 multidrug resistant strains were taken and MIC was performed with curcumin (50mg/L) with and without selected drugs.

Results : Significant reduction in MIC was observed after adding curcumin (50mg/L) with selected antimicrobial agents in 9/30 (30%) of multi drug resistant (MDR) isolates of *Pseudomonas aeruginosa*, while no change in MIC was observed when curcumin (50mg/L) was used alone, indicating its efflux pump inhibitor activity.

Conclusion: This study suggests role of efflux pump in development of drug resistance which can be overcome by use of an efflux pump inhibitor, with more emphasis on compound like curcumin which will have less or no adverse effects if used in vivo.

Keywords: Curcumin, Efflux pump, Multidrug resistance, Non-fermenters, Phe-arg-beta-naphthylamide

INTRODUCTION

Pseudomonas aeruginosa is increasingly recognized as an emerging opportunistic pathogen especially in hospital settings and well known for its drug resistance. The intrinsic multidrug resistance of *Pseudomonas aeruginosa* is a result of combination of factors which include an outer membrane with low permeability and the expression of efflux systems [1]. Efflux means transport of a substance out of cell. Multidrug resistance (MDR) can be defined as resistance to three or more antimicrobial classes [2]. Efflux pumps attribute to multidrug resistance by extruding various classes of antimicrobial agents and various other substances such as dyes, detergents, biocides and molecules required for cell to cell signalling [3,4]. Bacterial efflux systems generally fall into five classes, the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family [5].

Recently, it was observed that the use of efflux pump inhibitors (EPIs) is helpful to potentiate the activity of antimicrobial agents as many efflux pumps possess structural homology with them [6]. Hence to develop an effective EPI, there is a need to understand the basic structural and physiological mechanisms of the efflux pumps associated with drug resistance [7]. The mechanism of action of EPIs is through competitive inhibition where these become the substrate of efflux pumps instead of the target antibiotics. As long as these inhibitors are extruded outside the cells by the efflux pumps, the concentration of antibiotic increases intracellularly, eventually leading to cell death. Peptidomimetic compounds with phenylalanine arginyl beta naphthylamide (PAβN) also known as MC-207,110 have been used as broad spectrum EPI for *Pseudomonas aeruginosa* [8,9]. In addition to inhibition of efflux pump activity, PAβN permeabilizes bacterial membranes [10]. Among other synthetic compounds, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP) and verapamil has been investigated as potential inhibitor of efflux pump activity in *Pseudomonas aeruginosa* and Mycobacterium tuberculosis [8,11].

Curcumin is a natural extract derived from rhizomes of the plant *Curcuma* longa (Zingiberaceae) grown in regions of India and South East Asia. Extensive research in this field has shown its activity as an anti-inflammatory, antiviral, antioxidant, and anticancer agent[12-16]. It has been observed that curcumin potentiated the effect of various antibiotics against *Staphylococcus aureus* and reduced the virulence in animal pathogenicity models [17-18].

Among other natural compounds, plant alkaloid reserpine was shown in a study as an efflux pump inhibitor of *Bacillus subtilis* and *Streptococcus pneumoniae* [19,20]. Other studies have also identified a group of cationic berberine alkaloids as a potential efflux pump inhibitor [21]. For the first time, in present study, we investigated the role of curcumin as EPI by observing decrease in MIC values of selected antimicrobial agents against multidrug resistant (MDR) *Pseudomonas aeruginosa* strains tested and compared its activity with a known synthetic EPI.

MATERIALS AND METHODS

A total of 170 isolates of *Pseudomonas aeruginosa*, from clinical samples were processed in the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University from June 2011 to May 2012. Antimicrobial susceptibility testing was done by modified Kirby Bauer method with meropenem (10µg/disc) (Astra Zenica Pharma India), carbenicillin (100µg/disc), ceftazidime (30µg/disc), gentamicin (10µg/disc) and ciprofloxacin (5µg/disc) Hi–Media, Mumbai, India. Further MIC testing was carried out by agar dilution method.

Amount of antibiotic solution added (µl)† (Original stock solution concentration of 10mg/ml)	Volume of Mueller Hinton agar (ml)	Final concentration of antibiotic in 20 ml of medium used in each plate (µg/ml)
8192	11.808	4096
4096	15.904	2048
2048	17.952	1024
1024	18.976	512
512	19.488	256
256	19.744	128
128	19.872	64
64	19.936	32
32	19.968	16
16	19.984	8
8	19.992	4
4	19.996	2

[Table/Fig-1]: Preparation of antibiotic dilution range

Preparation of agar dilution plates: A series of dilution of antibiotics ranging from 2048 µg/ml to 2µg/ml were made from stock solution in Mueller Hinton agar plates as shown in [Table/Fig-1], which was allowed to equilibrate in water bath at 48°C- 50°C. The agar and antibiotics were mixed thoroughly and poured into petri dish in a depth of 4mm. The agar was allowed to solidify at room temperature and stored at 4–8°C. *Pseudomonas aeruginosa* ATCC (27853) was used as control.

INTERPRETATION

The results were interpreted according to the CLSI guidelines 2013, for MIC break points to be sensitive, intermediate and resistant [22].

MIC of antimicrobial agents with PAβN: Two different concentrations of PABN were taken to check the appropriate concentration for this study. PAβN (M.P biomedicals India) (20 mg/L and 50mg/L) [8], along with each antibotic of appropriate dilutions (ranging from 4096 µg/ml to 2 µg/ml), were added to the corresponding amount of molten Mueller Hinton agar. This was allowed to equilibrate in water bath at 48°–50°C. Media was poured in petridishes, allowed to settle and MIC testing were carried out on it.

MIC of antimicrobial agents with curcumin: Curcumin (Hi Media, Mumbai, India) was dissolved in dimethyl sulfoxide (Sigma Aldrich, India), and a stock concentration of 10 mg/ml was stored at -20°C. Final test concentrations consisted of 50, 30, 20, 15, 10, and 5µg/ml of curcumin solution [23]. Appropriate dilutions (ranging from 4096 µg/ml to 2 µg/ml) of each antimicrobial solution were added to the corresponding amount of molten Mueller Hinton agar which was allowed to equilibrate in water bath at 48° – 50°C along with curcumin at concentration mentioned above to determine its EPI activity. Mueller Hinton agar was prepared even with variable concentrations of curcumin, without antimicrobial, so as to find whether it has any antimicrobial effect when used alone.

RESULTS

Out of total 170 strains, 86 (50.58%) were resistant to atleast one antimicrobial agent tested and 30 (17.64%) were observed to be resistant against all the selected antimicrobial agents namely meropenem, carbenicillin, gentamicin, ceftazidime and ciprofloxacin according to MIC studies. It was observed that after increasing the concentration of PAβN from 20mg/L to 50 mg/L with those selected antimicrobials namely meropenem and carbenicillin the increase in sensitivity was only 3% and 13.33% respectively, which was not significant (p = 0.77). So the concentration of 20mg/L was considered appropriate for the present study.

Name of antimicrobial agent	Total no of sensitive strains after MIC testing % n=170	Total no of resistant strains after MIC testing %	Total number of sensitive strains after addition of PAβN (%)	Number of strains showing resistance due to efflux pump(%)
Meropenem	120(70.58%)	50(29.41%)	133(78.23%)	13(13/50=26%)
Carbenicillin	109(64.44%)	61(35.88%)	121(71.17%)	12(12/61=19.67%)
Ceftazidime	99(58.23%)	71(41.76%)	114(67.05%)	15(15/71=21.12%)
Gentamicin	83(48.82%)	87(51.17%)	96(58.77%)	13(13/ 87=14.9%)
Ciprofloxacin	74(43.52%)	98(57.64%)	83(48.82%)	9(9/98=9.18%)

[Table/Fig-2]: Susceptibility pattern of *Pseudomonas aeruginosa* as determined by MIC using selected antimicrobial agents with and without adding PAβN Note: As per CLSI guidelines 2013 for meropenem and gentamicin: Sensitive ≤ 4µg/ml; Intermediate susceptibility 8µg/ml; Resistance ≥ 16µg/ml; for carbenicillin: Sensitive ≤ 128 µg/ml; Intermediate susceptibility 256 µg/ml; Resistance ≥ 512 µg/ml; for ceftazidime: Sensitive ≤ 8µg/ml; Intermediate susceptibility 16µg/ml; Resistance ≥ 32µg/ml; for ciprofloxacin: Sensitive ≤ 1µgm/ml; Intermediate susceptibility 2µgm/ml; Resistance ≥ 4µgm/ml. Note: In this data intermediate susceptibility was considered as sensitive

Antimicrobial agent (µg/L)	Curcumin	Total no of resistant strains after MIC testing %
Meropenem	5	0
8-16	10	0
	15	0
	20	3
	30	3
	50	5

[Table/Fig-3a]: Multi drug resistant strains which became sensitive after adding curcumin with antimicrobial agents (meropenem) at its various concentrations

Antimicrobial agent (µg/L)	Curcumin (µg/L)	No of sensitive strains
Carbenicillin	5	0
16 - 64	10	0
	15	1
	20	3
	30	5
	50	8

[Table/Fig-3b]: Multi drug resistant strains which became sensitive after adding curcumin with antimicrobial agents (carbenicillin) at its various concentrations

Isolates which were resistant earlier and after addition of PAβN (20 mg/L) whose MIC dropped within the sensitive range, were considered to have efflux pump mediated resistance mechanism [Table/Fig-2]. No major effect of curcumin with antibiotics was observed till 15µg/ml but when concentration was increased from 20- 50µg/ml a considerable increase in sensitivity of strains was noted [Table/Fig-3a-e]. Best results were obtained at concentration of 50µg/ml which has been included in our study. None of the 30 MDR strains including ATCC strain of *Pseudomonas aeruginosa* was found susceptible to curcumin alone at any of the above concentration.

On interpretation of results, the EPI activity of curcumin was seen best with carbenicillin, followed by ceftazidime and meropenem. Finally out of the 30 drug resistant strains 9 became sensitive after adding curcumin (50µg/ml) with one or the other antimicrobial agents [Table/Fig-4].

Antimicrobial agent (µg/L)	Curcumin	Total no of resistant strains after MIC testing %
Ceftazidime	5	0
8-16	10	0
	15	2
	20	2
	30	5
	50	6

[Table/Fig-3c]: Multi drug resistant strains which became sensitive after adding curcumin with antimicrobial agents (gentamicin) at its various concentrations

Antimicrobial agent (µg/L)	Curcumin	Total no of resistant strains after MIC testing %
Gentamicin	5	0
8-16	10	0
	15	0
	20	1
	30	1
	50	3

[Table/Fig-3d]: Multi drug resistant strains which became sensitive after adding curcumin with antimicrobial agents (ceftazidime) at its various concentrations

Antimicrobial agent (µg/L)	Curcumin	Total no of resistant strains after MIC testing %
Ciprofloxacin	5	0
2-4	10	0
	15	0
	20	0
	30	1
	50	3

[Table/Fig-3e]: Multi drug resistant strains which became sensitive after adding curcumin with antimicrobial agents (ciprofloxacin) at its various concentrations

DISCUSSION

Pseudomonas aeruginosa is an important pathogen frequently associated with healthcare associated infections, particularly in critically ill or immunocompromised patients. The true prevalence of MDR *Pseudomonas aeruginosa* is not yet well established. However, rates of resistance increased for imipenem, quinolones by 15-23%, 15-32% and for third generation cephalosporins 16-25% [24].

A recent study showed that PAβN may be used for phenotypic screening for presence of efflux pump activity in clinical isolates of *Pseudomonas aeruginosa* along with genotypic methods [25]. Further, another study observed 4 to 8-fold reduction of meropenem MICs among *Acinetobacter baumanii* using PAβN as efflux pump inhibitor [26].

Among 170 strains of *Pseudomonas aeruginosa* included in the present study, antimicrobial resistance was observed to be contributed by efflux pump for one drug or the other drug in 27% (23/85) strains. Further among 30 MDR isolates, curcumin was responsible for reduction of MIC of 30% (9/30) of MDR *Pseudomonas aeruginosa* isolates to the level of susceptibility towards one or the other 5 drugs employed. In the present study 3 resistant isolates to gentamicin and ciprofloxacin became sensitive after adding curcumin and not after adding PAβN. The above observation indicates that curcumin is inhibiting the expression of efflux pump which are not inhibited by PAβN. This indicates the potential use of curcumin as an adjunct to antimicrobials in treatment of drug resistant bacterial infection.

Name of antimicrobials	Strains which became sensitive after adding PAβN(20µg/ml) n=30	Strains which became sensitive after adding Curcumin (50µg/ml)
Meropenem	7 (23.33%)	5 (16.6%)
Carbenicillin	2 (6.6%)	8 (26.66%)
Ceftazidime	6 (20%)	6 (20%)
Gentamicin	0 (0%)	3 (10%)
Ciprofloxacin	0 (0%)	3 (10%)

[Table/Fig-4]: Multi drug resistant strains which became sensitive after adding PAβN and curcumin

CONCLUSION

The mechanism by which curcumin has potentiated the effect of antimicrobial compounds in the present study appears to be due to inhibition of bacterial efflux pump which needs further study for its genotypic validation.

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