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Overcoming *Pseudomonas aeruginosa* Resistance Caused by Glycocalyx with Tobracef

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Abstract

The present study was aimed to compare the binding ability of selected chemicals (adjuvants) in 23 glycocalyx positive *Pseudomonas aeruginosa* isolates. Fractional inhibitory concentration indexes (FIC_{index}) and drug uptake study were conducted using selected adjuvants. Whole-cell alkaline phosphatase assay was used for assessment of outer membrane permeability. FIC_{index} was calculated using a microdilution checkerboard method.

Among the drugs tested, Tobracef was found to be the most effective against all the selected clinical isolates with minimum inhibitory concentrations (MICs) of about 8-16 µg/ml. Tobracef without adjuvants showed drug uptake of approximately 85.3%. When Tobracef was combined with either adjuvant CH1 or adjuvant CH2 the drug uptake was increased to 90.6% and 94.8% respectively but this increase was not statistically significant (P>0.05). The drug uptake of other comparator drugs including ceftazidime, tobramycin, amikacin, gentamycin, ceftazidime plus amikacin without adjuvants varied from 14 to 34%. Addition of adjuvant CH2 and adjuvant CH1 with these drugs enhanced the drug uptake by 8 to 11% and 11 to 22%, respectively which was statistically significant (P<0.01 with adjuvant CH2 and P<0.001 with adjuvant CH1). Tobracef without adjuvants showed maximum outer membrane permeability with 8.5 ± 0.70 permeability index at 8 hrs. Addition of adjuvant CH2 or adjuvant CH1 into Tobracef produced 9.1 ± 0.71 and 9.5 ± 0.98 permeability index which was statistically non-significant (P>0.05). Other comparator drugs exhibited a very low (only 2.4 to 3.5) permeability index and incorporation of adjuvant CH2 and adjuvant CH1 enhanced significantly the permeability index (P<0.01 with adjuvant CH2 and P<0.001 with adjuvant CH1). Interestingly, all the drugs showed additive effects when tested with both the adjuvants. However the exact mechanism of high membrane permeability of Tobracef is not known which needs to be explored. Thus, Tobracef is effective in dealing resistance from *P. aeruginosa* by increasing permeability and susceptibility towards *P. aeruginosa*.

Keywords: Glycocalyx; Divalent ions; *Pseudomonas aeruginosa*; Clinical isolates

Introduction

Pseudomonas aeruginosa, an opportunistic gram negative pathogen, continues to be a major cause of both hospital and community acquired infections and constitutes approximately 10% of the hospital infections [1,2]. It is thought to be the key pathogen in patients with immune suppression, cystic fibrosis and malignancy [3]. It has been reported that more than 90% of the deaths among cystic fibrosis are caused by *P. aeruginosa* [4].

A characteristic feature of *P. aeruginosa* strains is production of glycocalyx, also coined as capsule when it is gelatinous in nature and slime layer when irregularly diffused, which surrounds the cells and provides a certain degree of protection for its inhabitant against environmental threats including antibiotics [5]. Glycocalyx is primarily composed of uronic acids and carbohydrates [6,7] in both planktonic and biofilm cells. Glycocalyx, due to its polyanionic nature, can bind cationic antibiotics such as the aminoglycosides and thus restrict their diffusion by forming a diffusion barrier to the antimicrobial agents [8]. Amongst third generation cephalosporins, ceftazidime and amongst aminoglycoside, tobramycin are the drugs used to treat Pseudomonal infections. But in recent past, Pseudomonal resistance to these drugs has increasingly been reported [3,9,10]. It has been found that bacteria with glycocalyx exhibit enhanced resistant to antimicrobial agents [11,12]. Among the various types of mechanism of resistance, impermeability due to glycocalyx formation is the most prevalent in *P. aeruginosa*. Up to 90% of the *P. aeruginosa* isolates which are normally aminoglycoside resistant, appear to carry the impermeability type resistance [4]. It has been demonstrated that *P. aeruginosa* with glycocalyx showed a 49% decrease in permeability of tobramycin inside the bacterial membrane from 71% to 22% [13]. It has been shown that low outer membrane permeability is the root cause of all forms of resistance in *P. aeruginosa* [8]. The glycocalyx attracts divalent cations such as calcium and produce calcium complex which acts as a barrier to antimicrobial agents [14]. Thus, there is a need of an agent that breaks down the outer-membrane permeability barriers to make bacterial cells more susceptible to antibiotics [15]. It is contemplated that blocking of calcium ions may prevent the formation of glycocalyx thus use of chemicals/adjuvants with binding/blocking property together with antibiotics may be helpful in the treatment of infections caused by such type of bacteria.

Looking at the challenges posed by resistant *P. aeruginosa* to the clinicians, we aimed to compare the binding ability of selected chemicals, herein after termed as adjuvants, at different concentrations that increase bacterial cell membrane permeability which can be used together with antibiotics to fight infections caused by multidrug resistant glycocalyx positive *P. aeruginosa* isolates. Furthermore, we

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also studied the drug uptake to examine the quantity of drugs inside the outer membrane of glycocalyx forming *P. aeruginosa*.

Materials and Methods

Drugs

The drugs used for the study were as follows: Tobracef (ceftazidime+tobramycin), tobramycin, amikacin, gentamycin, ceftazidime, ceftazidime along with amikacin. All these were purchased from Indian market on behalf of sponsor.

Bacterial strain

A total of 23 multidrug resistant clinical isolates of *P. aeruginosa* obtained from different parts of India were included in the study. These isolates were further re-confirmed using conventional biochemical methods [16]. *P. aeruginosa* ATCC 27853 was used as a control strain. Prior to use, the isolates were inoculated on Soyabean casein digest broth medium (SCDBM, Hi-Media, Mumbai, India), and incubated at 37°C for overnight. The overnight grown cultures were then adjusted to 10^6 cfu/ml matching the turbidity standard of 0.5 Mac-Farland standards with SCDBM.

Glycocalyx characterization

In order to characterize the isolates having the capability to produce glycocalyx, all the clinical isolates were subjected to qualitative test for glycocalyx production as described previously [17].

Adjuvants

All of the adjuvants, such as CH1, CH2, (protected as trade secret), ascorbic acid, citric acid, disodium uridine monophosphate, and boric acid were obtained from Hi-Media (Mumbai, India) and reconstituted with water for injection. Working solutions were prepared using MH (Mueller Hinton, Himedia, Bombay, India) broth.

Minimum inhibitory concentration (MIC)

MIC testing was performed on planktonic cultures using the two fold dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The MIC value represents the lowest dilution at which bacteria fail to grow.

Fractional inhibitory concentration (FIC) study

In vitro drug interaction was determined by the checkerboard method as described elsewhere [19] and results were analyzed with the FIC_{index}. Growth control wells containing medium were included in each plate. Each test was performed in triplicate. The concentration of antibiotics needed to inhibit growth was recorded. The following formula was used to calculate FIC:

$$FIC = \frac{\text{MIC of drug in combination}}{\text{MIC of drug alone}}$$

The FIC_{index} (ΣFIC) calculated as the sum of each FIC, was interpreted as follows: synergy is defined as a FIC_{index} of ≤ 0.5. Antagonism is defined as a FIC_{index} of ≥ 2. An indifferent/additive effect is defined as a FIC_{index} of >0.5 to 2 or a micro dilution decrease of 1 dilution in the MIC of the one or the other drug or no change in the MIC of either of the drugs.

Effect of adjuvants on binding

Complexometric titration method was used to evaluate the chelating ability of each of adjuvant as described previously [20]. To assess the binding ability of adjuvants on Ca^{2+} and Mg^{2+} present in bacterial cells, a range of concentrations of adjuvants varying from 1 to 150 mM was used. Briefly, to separate conical flasks, 50 ml of bacterial culture containing various concentrations of adjuvants were added and placed all these conical flasks in incubator shaker at 37°C with rotating speed of 150 rpm. From each flask, an aliquot of 5 ml was withdrawn at time intervals of 2, 4, 6, 8, 10, and 12 hrs. All aliquots were centrifuged at 7000 rpm to pellet the cells and supernatant was used for total alginate assay. The pellets were suspended in 2 ml of phosphate buffer saline (PBS, pH 7.2). One ml of each cell suspension was added to 50 ml of conical flasks containing 6.0 ml of 0.5 M adjuvant CH1, 5 ml titration buffer (combination of 7.0% ammonium chloride and 12.5% ammonia solution, pH 10.5) and 5 ml of distilled water. After addition of one drop of Eriochrome black T indicator, the solution was titrated against 0.25 M $MgCl_2$ until the appearance of pink color. A control sample was also run simultaneously to compare the binding ability of adjuvants in comparison to control. Results are presented as percentage of binding of divalent compared to total divalent ions.

Total alginate analysis

The supernatant obtained after treatment was used to analyze the released alginate comprising of uronic acid and carbohydrate by carbazole assay [21]. The optical density (OD) was read at 530 nm. D-glucuronic acid standard of 1 to 40 µg/ml was prepared.

Drug uptake study

Drug uptake study was done by quantifying the amount of amines present in the sample as described earlier [22]. For experiment, 50 ml of bacterial samples (10^6 cfu/ml) were treated with drugs at their half of MIC containing adjuvants and 5 ml aliquot was withdrawn at different time intervals of 2, 4, 6, 8, 10 and 12 hrs from each treatment group. These aliquots were centrifuged at 7000 rpm for 5 min and pellets were suspended in 2 ml of 10 mM PBS. One ml of each bacterial suspension was added to tubes containing 1.0 ml of ninhydrin reagent (1% w/v) and 0.1 ml of pyridine and vortexed immediately. All these tubes were then immediately placed at 70°C for 15 min. After 15 min, tubes were cooled on ice-water bath and PBS was added to bring the total volume up to 12.5 ml. Absorbance was measured at 400 nm. Controls were also run simultaneously.

Permeability assay

Whole-cell alkaline phosphatase assay for assessment of outer membrane permeability is carried out by method detailed by Wang et al. [23]. An overnight culture of all seventeen selected *P. aeruginosa* clinical isolates grown in SCDBM were treated with antibiotics at half MIC concentration and processed as described by Wang et al. [23]. An index of outer membrane permeability was calculated as A410/A600(P) where P is the concentration of para-nitrophenyl substrate.

Statistical analysis

Results are expressed as mean ± SD values. Statistical evaluations were carried out using one way analysis of variance (ANOVA) followed by tuke'kramer multiple comparison between control and treatments groups. A value of *P<0.05; **P<0.01; ***P<0.001, compared to controls was considered significant.

Results

Strain characterization and glycocalyx identification

All the isolates were confirmed to be *P. aeruginosa*. Out of the 23 *P.*

aeruginosa isolates, 17 (74%) were positive for glycocalyx production. These glycocalyx positive isolates were used for further study.

MIC

Among the drugs tested, Tobracef was found to be the most effective against all the selected clinical isolates with MIC values of about 16-32 µg/ml. One fold decrease in MIC was found when Tobracef was incorporated with both adjuvant CH2 and adjuvant CH1. The MICs for ceftazidime and amikacin were 512-1024 and 64-128 µg/ml, respectively. For each of tobramycin and gentamycin, MICs ranged 256-512 µg/ml. The MIC values for ceftazidime plus amikacin were 128-256 µg/ml. Surprisingly, when MICs of these drugs were tested with the best performing adjuvants adjuvant CH2 and adjuvant CH1, only one and two fold decrease in MIC was observed, respectively (Table 1).

Effect of adjuvants on binding of divalent ions

As expected, among the tested adjuvants adjuvant CH1and adjuvant CH2 had higher binding property compared to others. Binding ability of adjuvant CH1 increased with increasing the concentration and reached maximum at 10 mM. Further increase in the concentration of adjuvant CH1 failed to yield significant binding activity. We observed that the binding ability of adjuvant CH1 increased with time and found to be maximum at 8 hrs and thereafter remained almost constant up to 12 hrs (Figure 1A). Adjuvant CH2 also demonstrated binding activity with increasing concentration and exhibited maximum binding at 30 mM adjuvant CH2 and thereafter remained constant (Figure 1B). Interestingly, adjuvant CH2 also showed maximum binding at 8 hrs and then became constant. Adjuvant CH1 was found to be the most effective in producing highest binding ability at about three times lesser concentration of adjuvant CH2. The other adjuvants including ascorbic acid, citric acid, disodium uridine monophosphate, and boric acid failed to produce significant binding ability upto 150 mM (data not shown).

Effect of adjuvants on alginate reduction

Drugs	*P. aeruginosa* (MIC µg/ml)		
	Without adjuvant	CH1	CH2
Ceftazidime	512-1024	128-256	256-512
Tobramycin	256-512	64-128	128-256
Amikacin	64-128	16-32	32-64
Gentamicin	256-512	64-128	128-256
Ceftazidime + Amikacin	128-256	32-64	64-128
Tobracef	16-32	8 to16	8 to 16

Table 1: MICs for antibacterial agents against *P. aeruginosa* clinical isolates.

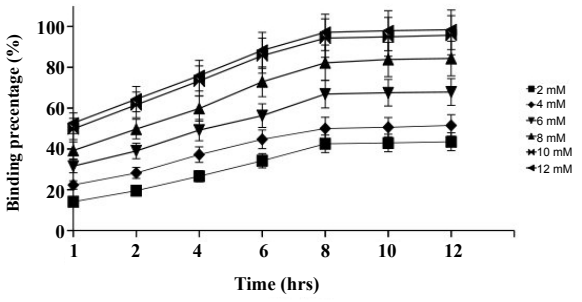

Figure 1a: Binding percentage of divalent ions by CH1.

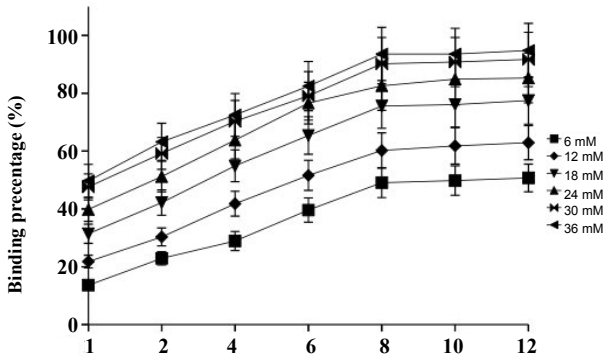

Figure 1b: Binding percentage of divalent ions by CH2.

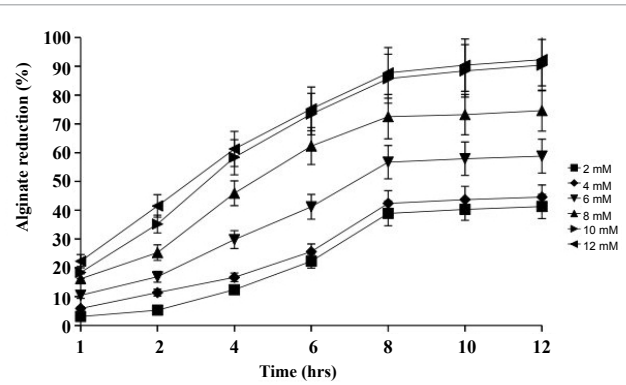

Figure 2a: Alginate reduction after CH1 treatment.

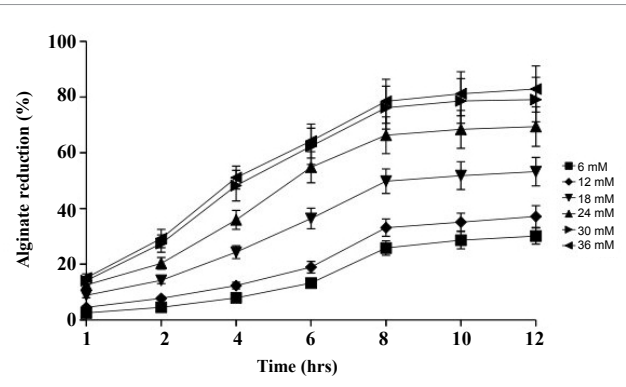

Figure 2b: Alginate reduction after CH2 treatment.

As shown in Figure 2A and 2B, alginate levels reduced with increasing the concentrations of adjuvant CH1 and adjuvant CH2. Up to 85% and 76% of alginate was reduced at 8 hrs when glycocalyx positive clinical isolates were treated with 10 mM adjuvant CH1 and 30 mM adjuvant CH2. Further, increasing the concentration of both of these adjuvants did not produce any significant reduction in alginate. However, the other adjuvants such as ascorbic acid, citric acid, disodium uridine monophosphate, and boric acid did not yield any pronounced reduction in alginate upto 150 mM.

FIC index

FICindex was conducted using the optimised concentrations of adjuvant CH1 (10 mM) and adjuvant CH2 (30 mM) and results are

Drugs	FIC index	
	CH2	CH1
Ceftazidime	0.94 ± 0.07	0.87 ± 0.05
Tobramycin	0.93 ± 0.08	0.88 ± 0.06
Amikacin	0.95 ± 0.08	0.86 ± 0.07
Gentamicin	1.43 ± 0.09	1.12 ± 0.08
Ceftazidime + Amikacin	1.53 ± 0.1	1.13 ± 0.07
Tobracef	1.65 ± 0.1	1.34 ± 0.08

Synergy= FIC_{index} ≤ 0.5; antagonism FIC_{index} ≥ 2; additive FIC_{index} of >0.5 to 2.

Table 2: FIC_{index} between antibacterial agents and adjuvants for *P. aeruginosa*.

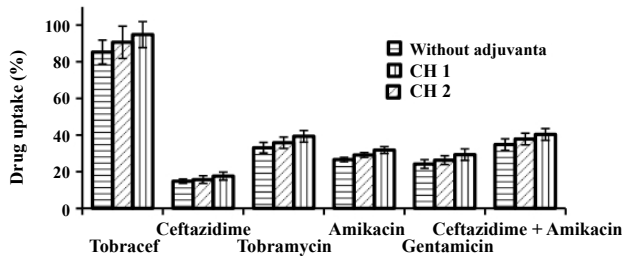

Figure 3: Comparison of drug uptake.

presented in Table 2. Results revealed that all the drugs with both adjuvants, adjuvant CH1 or adjuvant CH2 showed additive effect.

Drug uptake study using ninhydrin

After screening of the adjuvants and their concentrations which yielded highest binding, the optimized concentrations of adjuvant CH1 (10 mM) and adjuvant CH2 (30 mM) was incorporated with different drugs for uptake study. We have studied the drug uptake at 2, 4, 6, 8, 10 and 12 hrs time intervals and highest drug uptake was noted at 8 hrs so here we have presented the data of only 8 hrs. Our results showed that Tobracef without adjuvants showed drug uptake of approximately 85.3%. When Tobracef was combined with adjuvant CH1or adjuvant CH2 the drug uptake was increased to 90.6% and 94.8% respectively but this increase was not statistically significant (P>0.05). The drug uptake of other comparator drugs including ceftazidime, tobramycin, amikacin, gentamycin, ceftazidime plus amikacin without adjuvants varied from 14 to 34%. Addition of adjuvant CH2 and adjuvant CH1with these drugs enhanced the drug uptake by 8 to 11% and 11 to 22%, respectively which was statistically significant (P<0.01 with adjuvant CH2 and P<0.001 with adjuvant CH1). These results suggest that Tobracef uptake without adjuvants was highest and significantly significant (P<0.001) in comparison to other drugs in both cases, with or without adjuvants (Figure 3).

Permeability assay

To establish correlation between drug uptake and membrane permeability a whole cell alkaline phosphatase assay was performed and results were interpreted as outer membrane permeability index. An index of outer membrane permeability was calculated to know the penetration of drugs inside the bacterial membrane. Tobracef without adjuvants showed maximum outer membrane permeability with 8.5 ± 0.70 permeability index at 8 hours. Addition of adjuvant CH2 or adjuvant CH1 into Tobracef produced 9.1 ± 0.71 and 9.5 ± 0.98 permeability index which was statistically non-significant (P>0.05) suggesting incorporation of adjuvants did not further increase the permeability index. Ceftazidime without adjuvant showed the least outer membrane permeability with permeability index 1.5 ± 0.11. Addition of adjuvant CH2 and adjuvant CH1 increased the permeability index to 2.4 ± 0.21 and 2.6 ± 0.18 which was statistically significant P<0.01 and P<0.001, respectively. Other comparator drugs exhibited permeability index 2.4 to 3.5 and incorporation of adjuvant CH2 and adjuvant CH1 enhanced significantly the permeability index (P<0.01 with adjuvant CH2 and P<0.001 with adjuvant CH1) (Figure 3 and 4).

Discussion

Now-days, *P. aeruginosa* is considered a leading cause of gram negative bacterial infections especially in immuno-suppressed patients who need prolonged hospitalization [3]. The occurrence of glycocalyx in *P. aeruginosa* is well documented [3,24]. Our experiment demonstrated that treatment of bacterial cells with either of adjuvants adjuvant CH1or adjuvant CH2 resulted in significant reduction of alginate. The reduced alginate would sensitize multidrug resistant *P. aeruginosa* to the antibiotic by breaking the diffusion barrier. Earlier investigation also noted that removal of the alginate from bacterial cells changes the susceptibility of them towards drugs [25].

In addition, it has been shown that divalent metal ions such as calcium present in bacterial cell also contribute to antibiotic resistance [26-28]. Our results demonstrated that exposure of bacterial cells with either adjuvant CH1or adjuvant CH2 yielded a significant reduction of divalent ions from bacterial cells thus helping in enhancement of susceptibility. When either of these adjuvants was incorporated with drugs one to two fold reductions in MIC was observed. In contrast to other comparator drugs, Tobracef without adjuvants showed higher sensitivity to multidrug resistant clinical isolates of *P. aeruginosa* which is evident by 4 to 32 time lesser MIC in comparision to comparator drugs. These lower MICs of Tobracef is directly associated with higher permeability of drugs. Tobracef may have some kind of binding property which enhanced the uptake of this drug into bacterial cells and further addition of adjuvants with this drug failed to yield significant drug uptake.

Alkaline phosphatase assay was used to evaluate outer membrane permeability as it's a convenient tool to assess bacterial outer membrane permeability. Our data have demonstrated a positive correlation between the outer membrane permeability index and bacterial susceptibility to studied drugs in glycoclayx producing MDR *P. aeruginosa* strains. Addition of adjuvants with drugs significantly (P<0.01 to P<0.001) increased permeability index because adjuvants bind divalent ions as well as remove alginate thus enhancing the permeability. However, Tobracef without adjuvants exhibit very higher

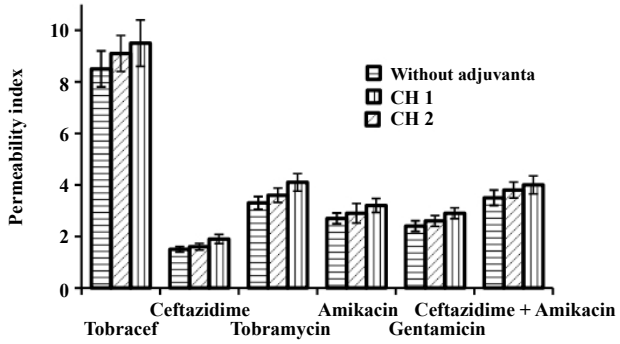

Figure 4: Comparison of bacterial outer membrane permeabilities.

permeability index when compared with other drugs. Incorporation of adjuvant CH2 and adjuvant CH1with Tobracef could not enhance significant permeability suggesting adjuvants failed to remove any more divalent ions or alginate from the bacterial cells and Tobracef itself is sufficient to give the activity which was several folds higher when compared with other drugs along with adjuvants because of synergistic activity.

Conclusion

Our results clearly showed that among the tested drugs Tobracef was more effective in multidrug resistant *P. aeruginosa* as evident by lesser MIC value. Also Tobracef demonstrated enhanced drug uptake and higher outer membrane permeability index compared to other drugs. However the exact mechanism of Tobracef how this causes these effects are not known which needs to be explored. Thus, Tobracef is effective in dealing resistance from *P. aeruginosa* by increasing permeability and susceptibility towards *P. aeruginosa*.

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