EFFECTS OF PSYCHOTROPIC DRUGS AS BACTERIAL EFFLUX PUMP INHIBITORS ON QUORUM SENSING REGULATED BEHAVIORS

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ABSTRACT
Psychotropic drugs are known to have antimicrobial activity against several groups of microorganisms. The antidepressant agents such as duloxetine, paroxetine, hydroxyzine and venlafaxine are shown to act as efflux pump inhibitors in bacterial cells. In order to the investigation of the effects of psychotropic drugs were determined for clinically significant pathogens by using standart broth microdilution method. The anti-quorum sensing (anti-QS) activity of psychotropic drugs was tested against four test pathogens using the agar well diffusion method. All drugs showed strong inhibitory effect on the growth of S. typhimurium. Additionally, quorum sensing-regulated behaviors of Pseudomonas aeruginosa, including swimming, swimming and twitching motility and alkaline protease production were investigated. Most effective drugs on swimming, swimming and twitching motility and alkaline protease production production, respectively, were paroxetine and duloxetine; hydroxyzine and venlafaxine; paroxetine and venlafaxine; venlafaxine. Accordingly, psychotropic drugs were shown strongly anti-QS activity by acting as bacterial efflux pump inhibitors and effection on motility and alkaline protease production of P. aeruginosa.

Keywords: Anti-quorum sensing activity, efflux pump, motility, psychotropic drugs, P. aeruginosa

INTRODUCTION
Efflux bacterial systems involved in quorum sensing (QS) contribute to multidrug resistance as they expel different types of antibiotics and chemicals such as dyes, organic solvents, detergents, molecules needed for the cell to cell communication, biocides, and metabolic products. Hence understanding the mechanisms by which these pumps act and how to overcome its activity opens the door for restoring the antibacterial activity and constitute a promising target for novel antibacterial agents (Vidal et al., 2009; Dreier, 2007; Piddock, 2006; Schweizer, 2003). The continuous increase in the development of multidrug resistance by many pathogens has resulted in difficulties fighting many infectious diseases. In view of the fact that the majority of those multidrug resistant pathogens expresses and overproduces efflux pumps that are responsible for the expelling and extruding of the antibiotics from inside the cells, the new direction for other chemotherapeutics is the use of efflux pump inhibitors (Page's and Amaral, 2009). Effection of bacterial quorum sensing (QS) systems could be an effective alternative in as much as they regulate a broad spectrum of cell functions, including virulence factor production and motility. Efflux bacterial systems are known related to quorum sensing. The recent research focus is to develop antipathogenic agents to control bacterial diseases by inhibiting QS (Adonizio et al., 2008; Al-Hussaini and Mhasneh, 2009).

The pathogenic bacterium Pseudomonas aeruginosa uses quorum sensing systems to regulate genes controlling virulence, motility and biofilm formation. P. aeruginosa has three modes of surface motility: swarming (Overhage et al., 2008; Verstraeten et al., 2008), twitching (Semmler et al., 1999; Skerker and Berg, 2001), and swimming (Murray and Kazmierczak, 2008). Twitching and swarming motilities are strongly linked with biofilm development and pathogenesis, and their mechanical components, the flagellum and type IV pilus (TFP), have been demonstrated to be virulence factors (Overhage et al., 2008; Murray and Kazmierczak, 2008). Swarming, swimming and twitching are strongly related to disease (Allison et al., 1992; Elvers and Lappin-Scott, 2000). Bacterial proteases have the potential to destroy the structural and functional proteins that constitute host tissues as well as to destroy proteins important in host defense. Expression of many of the virulence factors (including LasB elastase, LasC protease, alkaline protease) in P. aeruginosa is controlled by quorum sensing (QS) system (Venturi, 2006). Antimicrobial activity was described in some psychotropic drugs (Cederlund et al., 1993; Kristiansen, 1990; Kristiansen, 1992). The studies show that these psychotropic drugs, namely sertraline, fluoxetine and paroxetine have a significant antibacterial activity, mainly against several groups of pathogen bacteria and they may act as efflux pumps inhibitors since they also act on human cells and bacterial cells as pump inhibitors (Munoz-Bellido et al., 2002). Psychotropic drugs are capable of inhibiting slime production in coagulase-negative staphylococci (Mun‘oo’ Créado et al., 1997), and inhibit swarming in Proteus (Mun‘oo’ Créado et al., 1998). The aim of this study was to determine the anti-QS activity, effects of psychotropic drugs on efflux pump of clinically important pathogens and QS-controlled cellular functions including motility and alkaline protease production of P. aeruginosa.

MATERIAL AND METHODS
Bacterial strains, growth and media conditions
Test pathogens including Vessoria enterolitica (ATCC 9610), Staphylococcus aureus (ATCC 25923), Salmonella typhimurium (ATCC 14028), Pseudomonas aeruginosa (ATCC 35032) were maintained in 2% Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% (w/v) NaCl) and on LB agar (supplemented with 1% (w/v) agar) prior to inoculation into the motility assays. Swarn, swim, and twitch media were prepared to assess the corresponding type of motility. Swarn medium consisted of 8 g/L nutrient broth and 0.5% (w/v) agar supplemented with D-glucose (5 g/L, filter sterilized and added separately). Swarn and twitch media consisted of LB broth supplemented with 0.3% and 1.0% (w/v) agar, respectively (Wiegand et al., 2008). Alkaline protease medium were consisted of LB broth supplemented with 1.5% (w/v) agar and 2% (w/v) skim milk. For minimal inhibitory concentration (MIC) assay were used Mueller Hinton broth (MHB). All culture strains were incubated at 37°C for 24 hours. Psychotropic drug stock solutions were prepared by dissolving duloxetine, paroxetine, hydroxyzine and venlafaxine in distilled water (100, 75, 50, 37.5, 25, 18.75, 12.5, 9.375, 6.25 mM ), followed by heating to 100°C and filter sterilization using 0.2 μm pore size filters.

Minimal inhibitory concentration (MIC) assay
MIC was assessed using the standard broth microdilution protocol as described by Wiegand et al. (2008) with a change in incubation time from 20 hour to 24 hour. Overnight cultures were grown in Mueller Hinton broth (MHB) at 37°C and diluted to yield an inoculum of approximately 1 × 10^6 CFU/mL. Cultures were further diluted 1:100, and inoculated with 100, 75, 50, 37.5, 25, 18.75, 12.5, 9.375, 6.25 mM drug solutions in a polystyrene 96-well plates. Growth was done for 24 hour at 37°C. After growth, MIC value taken as the concentration that inhibited macroscopic growth was observed, and also determined by spectrophotometer.

**Agar well diffusion assay**

Agar well diffusion assay was used to detect anti-QS activity of the psychotropic drugs. Bacterial pathogens Y. enterolitica (ATCC 9610), S. aureus (ATCC 25923), S. typhimurium (ATCC 14028), P. aeruginosa (ATCC 35032) were used in this study. Cultures were grown and maintained on LB medium. Overnight cultures were prepared by diluting culture suspension in 0.9% saline to achieve an absorbance of 0.3 at 600 nm as determined by a spectrophotometer. A sterilized cotton swab was dipped in the diluted cultures and spread over the surface of the LB agar (Nathan et al., 1978). Wells were made in the inoculated agar medium under sterile conditions (7 mm in diameters), and 100 µl of each psychotropic drug determined MICs was loaded onto the well in LB plates. The plates were incubated 37°C for 24 hour and anti-QS was detected by measuring the clear zone in diameter.

**Motility and alkaline protease production**

Motility assays were undertaken in petri dishes. The swimming, twitching motilities, and alkaline protease production of P. aeruginosa were performed using swim, swim, twitch and alkaline protease plates. The MIC concentration of each psychotropic drug was added to plates prior to a 30–45 min drying period. For the swimming, swimming, twitching assays and inhibition of alkaline protease production, the bacterial cells were gently inoculated using a toothpick at the center of the agar surface (Deziel et al., 2003) and the plates were incubated at 25°C for 16 hour, 37°C for 16–18 hour, 30°C for 16 hour and 37°C for 24 hour, respectively, and halo diameters were measured. Data from swimming, swimming, twitching assays and alkaline protease production were used as control against zero inhibition exhibited on each media by no drug.

**RESULTS AND DISCUSSION**

Growth inhibition following psychotropic drugs treatment of pathogens, MICs were determined. MIC assay results were obtained with different concentrations of the duloxetine, paroxetine, hydroxyzine and venlafaxine. Respectively, it was determined as 75 mM, 100 mM, 75 mM, 100 mM for Y. enterolitica; 37.5 mM, 75 mM, 100 mM for S. aureus; 75 mM, 100 mM, 100 mM for S. typhimurium; 37.5 mM, 75 mM, 75 mM, 37.5 mM, 75 mM for P. aeruginosa. For further studies, these values were used for anti-QS activity against each pathogen bacteria, and also 37.5 mM concentration of the each psychotropic drugs was used on P. aeruginosa motility and alkaline protease production.

Four psychotropic drugs (duloxetine, paroxetine, hydroxyzine and venlafaxine) selected for their anti-QS activity were tested against four pathogens on LB plates. QS inhibition was detected by a clear zone, and the diameter of zone of inhibition was determined as 75 mM, 37.5 mM, 37.5 mM, 37.5 mM, 37.5 mM for P. aeruginosa. It was determined that for swimming, swimming and twitching motilities, respectively, the most effective drugs were paroxetine and duloxetine (Fig 2 [1b, 1d]), duloxetine (Fig 2 [2b]), paroxetine and venlafaxine (Fig 2 [3d,3e]). But significantly, small and twitch halo diameters of P. aeruginosa were very nearly inhibited in the presence of all drugs (Tab 2). As seen figure 3, swimming motility showed less inhibitory results than swimming and twitching motility, due to a lack of consistent replicates across three biological repeats. Also we observed smaller clear zones on alkaline protease plates that drug concentrations at 37.5 mM significantly effected alkaline protease production (Fig 2 [4]). The most effective drug on alkaline protease production was determined as venlafaxine (Fig 2 [4d], Fig 3).

**Table 1** Inhibition zones of psychotropic drugs against Y. enterolitica, S. typhimurium, S. aureus, P. aeruginosa. (*Most effective drug inhibition zones).

<table>
<thead>
<tr>
<th></th>
<th>Duloxetine</th>
<th>Paroxetine</th>
<th>Hydroxyzine</th>
<th>Venlafaxine</th>
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<tbody>
<tr>
<td>Y. enterolitica</td>
<td>30±</td>
<td>23</td>
<td>21</td>
<td>30±</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>30</td>
<td>30</td>
<td>35±</td>
<td>35±</td>
</tr>
<tr>
<td>S. aureus</td>
<td>32±</td>
<td>23</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>26±</td>
<td>24</td>
<td>24</td>
<td>33±</td>
</tr>
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</table>

Duloxetine, paroxetine, venlafaxine and hydroxyzine decreased the three types of motility of P. aeruginosa were observed in distinct decreases in halo diameter in the presence of drug concentrations at 37.5 mM (Fig 2 [1,2,3]). When comparing the percent inhibition effect of psychotropic drugs on swimming, twitching and swimming motility, it was seen much greater effect of drugs on swimming and twitching (Fig 5).

Swarming, swimming and twitching motility assays showed decreases at 37.5 mM MIC concentration of each drug and for P. aeruginosa. It was determined that for swimming, swimming and twitching motilities, respectively, the most effective drugs were paroxetine and duloxetine (Fig 2 [1b, 1d]), duloxetine (Fig 2 [2b]), paroxetine and venlafaxine (Fig 2 [3d,3e]). It was determined that for swimming, swimming and twitching motilities, respectively, the most effective drugs were paroxetine and duloxetine (Fig 2 [1b, 1d]), duloxetine (Fig 2 [2b]), paroxetine and venlafaxine (Fig 2 [3d,3e]). But significantly, small and twitch halo diameters of P. aeruginosa were very nearly inhibited in the presence of all drugs (Tab 2). As seen figure 3, swimming motility showed less inhibitory results than swimming and twitching motility, due to a lack of consistent replicates across three biological repeats. Also we observed smaller clear zones on alkaline protease plates that drug concentrations at 37.5 mM significantly effected alkaline protease production (Fig 2 [4]). The most effective drug on alkaline protease production was determined as venlafaxine (Fig 2 [4d], Fig 3).

**Figure 1** Anti-QS activities of four psychotropic drugs. (a) Yersinia enterolitica, (b) Salmonella typhimurium, (c) Staphylococcus aureus, (d) Pseudomonas aeruginosa. K:Control, D:Duloxetine, H:Hydroxyzine, P:Paroxetine, V:Venlafaxine.

**Figure 2** Inhibition of P. aeruginosa swimming (1), swimming (2), twitching (3) motility and alkaline protease activity (4) by psychotropic drugs (a:control, b:duloxetine, c:hydroxyzine, d:paroxetine, e:venlafaxine).

**Table 2** Pseudomonas aeruginosa swimming, swimming, twitching motility and alkaline protease zones in the presence of psychotropic drugs.

<table>
<thead>
<tr>
<th></th>
<th>Swimming</th>
<th>Swimming</th>
<th>Twitching</th>
<th>Alkaline protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60±</td>
<td>40±</td>
<td>50±</td>
<td>20±</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>4±</td>
<td>4±</td>
<td>6± 1,5</td>
<td>4± 0,5</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>3±</td>
<td>7± 0,5</td>
<td>5± 0,5</td>
<td>3± 1</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>5± 2</td>
<td>8± 1</td>
<td>3± 1</td>
<td>4± 1</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>5± 2</td>
<td>6± 1,5</td>
<td>2± 0,5</td>
<td>2± 0,5</td>
</tr>
</tbody>
</table>

129
Effects of psychotropic drugs on microorganisms is not limited to antimicrobial activity. They also act as efflux pump inhibitors (Munoz-Bello et al., 2002) and modify bacterial metabolism, as shown in studies on slime and swarming inhibition (Munoz’Criado et al., 1997). Our results show that psychotropic drugs have anti-QS properties that are mediated through their inhibition of efflux pumps that extrude the nosocomial QS signal before it reaches its intended target. Inhibition of QS activity is not caused by inhibition of growth; it can also be done by disruption of QS signalling systems without killing bacteria by using psychotropic drugs. Hentzer et al. (2002) demonstrated the use of a furanone compound as a QS inhibitor and have focussed on the effect the furanone exerts on the las QS sensing system. Furanone compounds have been shown to hold promise as AHL-antagonists and for development of novel non-antibiotic, anti-pathogenic agents, which interfere with bacterial cell-to-cell communication and render bacteria less virulent. Also, a focus of a study was the BpeAB-OprP pump. It would be prudent to study the roles of B. pseudomallei resistance-nodulation-diviision efflux pumps in the efflux of acyl-HSLs. In P. aeruginosa, reduced synthesis of signal molecules was associated with overexpression of the MexAB-OprM and MexEF-OprN efflux pumps (Kohler et al., 2001), while reduced production of quorum-sensing regulated products was associated with an impaired MexGHIOpmpD pump (Aendekerk et al., 2002). Inhibition of these efflux pumps would be therapeutically beneficial, because it attenuates virulence by preventing quorum sensing (Inoue et al., 2008). As expected in our study, psychotropic drugs inhibit the efflux pump of a pathogenic bacterium and may be exploited for the prevention of QS responses of infecting bacteria. P. aeruginosa is a model organism, but this could potentially extend to other organisms that undertake motility, such as Escherichia coli, Vibrio parahaemolyticus, Serratia marcescens, Salmonella enterica and Proteus mirabilis (Copeland et al., 2009). P. aeruginosa is known to exhibit movement on surfaces by three types of motilities: swimming, swarming, and twitching. It has been observed that the non-antibiotic psychotropic drugs such as duloxetine, paroxetine, venlafaxine and hydroxyzine hinders motility of P. aeruginosa. But the inhibition of swimming motility was not as drastic as that of swarming and twitching motility. The psychotropic drugs might have some effects on flagella-related processes, namely, flagella biosynthesis, rotation, and chemotaxis, which may lead to a decrease in swimming and swarming activities. Also, twitching motility, which is dependent on functional type IV pili (Mattick, 2002), was significantly affected by the addition of drugs, this result indicated that twitching inhibition by the drugs was caused by defects in pili-related functions. Since bacterial motility necessary for proper biofilm formation was impaired in a concentration-dependent manner, these data suggest that the suppressive effect of psychotropic drugs on flagella-related and pili-related motility. In swimming and twitching motilities, the cells move independently, but swarming requires the bacteria to effectively work together via a process termed “quorum-sensing,” involving bacteria sensing the extracellular signals produced by other bacteria (Tremblay et al., 2007). Given the importance of swimming motility for conferring biofilm development (O’Toole et al., 2000; Toutain et al., 2004) and antibiotic resistance (Ciaizza et al., 2005; Butler et al., 2010), on going research in our laboratory is aimed at investigating the effect of psychotropic drugs as efflux pump inhibitors. It was found that there was an inhibitory effect of drugs on swimming, swimming, and twitching motility in P. aeruginosa, but we cannot conclude whether or not drugs preferentially targets one mechanism or motility system in P. aeruginosa. It was found that a BCFA, antiseo-C15:0, inhibits swimming, swimming, colony wetness, and biofilm formation in P. aeruginosa (Inoue et al., 2008). Also, the swimming motility of P. aeruginosa was blocked by cranberry proanthocyanidins and other tannin-containing materials (O’May and Tufenkji, 2011). As cell motility are proven instrumental in biofilm formation, it was investigated whether or not salicylic acid affected the motility of P. aeruginosa. There was also a direct inhibitory effect of salicylic acid on swimming, swimming, and twitching motility in both PA01 and PA14 lab strains (Chow et al., 2011). The motility in this present study illustrated the effect as 37.5 mM MIC concentrations of drugs had significant effects in decreasing swimming, swimming and twitching motility for P. aeruginosa. P. aeruginosa secretes alkaline protease and two elastases (A and B) that have been characterized as exoenzymes and virulence factors (Sutter, 1994). Proteases are important in tissue damage caused by P. aeruginosa infection and P. aeruginosa as a pathogen use proteases to cross proteinaceous barriers within the host contributes to bacterial virulence. Production of proteases (elastase, LasA protease and alkaline protease) in P. aeruginosa is undercontrolled las quorum sensing system including biosynthesis of AHL molecule and the expression of LasA (LasA protease) and apr (alkaline protease) (Pesci et al., 1997). The alkaline protease activity at 37.5 mM MIC concentrations of drugs has been shown by smaller clear zone. QS system can be interfered with inhibition AHL molecule biosynthesis by QS inhibitors (Kumar et al., 2011). Therefore, we decided to use psychotropic drugs such as duloxetine, paroxetine, hydroxyzine and venlafaxine for the QS inhibitory activity. CONCLUSION Psychotropic drugs have effected bacterial efflux pump related to QS and suppressed alkaline protease production and swimming, swimming and twitching motility by blocking efflux pump and QS system. Thus, psychotropic drugs such as duloxetine, paroxetine, hydroxyzine and venlafaxine will be played active role as an alternative antimicrobial therapeutic on the treatment of infectious diseases. REFERENCES ADONZIO, A.L., KONG, K.F. AND MATTHE, K. 2008. Inhibition of quorum sensing-controlled virulence factor production in Pseudomonas aeruginosa by South Florida plant extracts. Antimicrobial Agents Chemistry, 52, 198–203, http://dx.doi.org/10.1128/AAC.00612-07 AENDEKER, S., GHYSELS, B., CORNELIS, P. AND BAYSSE, C. 2002. Characterization of a new efflux pump, MexGHIOpmpD, from Pseudomonas aeruginosa that confers resistance to vanadium. Microbiology, 148, 2371–2381. AL-HUSSAINI, R. AND MAHASNEH, A.M. 2009. Antibacterial and anti- quorum sensing activity of different parts of Laurus nobilis extracts. Journal Medical Journal, 43(4), 286–298. ALLISON, C., COLEMAN, N., JONES, P.L. AND HUGHES, C. 1992. Ability of Proteus mirabilis to invade human urethelial cells is coupled to motility and swimming differentiation. Infection and Immunity, 60, 4740–4746. BUTLER, M. T., WANG, Q. F. AND HARSEY, R. M. 2010. Cell density and mobility protect swarming bacteria against antibiotics. Proceedings of the National Academy of Sciences of the United States of America, 107, 3776–3781, http://dx.doi.org/10.1073/pnas.0910934107 CAIAZZA, N. C., SHANKS, R. M. Q. AND O’TOOLE, G. A. 2005. Rhamnolipids modulate swimming motility patterns of Pseudomonas aeruginosa. Journal of Experimental and Applied Microbiology, 187, 7351–7361, http://dx.doi.org/10.1128/IB.187.21.7351-7361.2005 CEDERLUND, H. AND MARDH, P.A. 1993. Antibacterial activity of non- antibiotic drugs. Journal of Antimicrobial Chemotherapy, 32, 355–65, http://dx.doi.org/10.1093/jac/32.4.355 CHOW, C. Y., KONG, C. F., LAM, Y. C. AND FOK, R. K. 2011. Salicylic Acid Affects Swimming, Twitching and Swarming Motility in Pseudomonas aeruginosa, resulting in Decreased Biofilm Formation. Journal of Experimental Microbiology and Immunology, 15, 22 – 29. COPELAND, M. F., AND WEIBEL, D. B. 2009. Bacterial swimming: a model system for studying dynamic self-assembly. Soft Matter, 5, 1174–1187, http://dx.doi.org/10.1039/B812146J DE ZIEL, E., LEPINE, F., MILLOT, S. AND VILLEMUR, R. 2003. rhlA is required for the production of a novel biosurfactant promoting swimming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanolic acids (HAA s), the precursors of rhamnolipids. Microbiology, 149, 2005–2013, http://dx.doi.org/10.1099/mic.0.26154-0 DREIER J. 2007. Active drug efflux in bacteria. B.A. Bonomo and M. Tolmasy (ed.), Enzyme-mediated resistance to antibiotics Washington, DC, ASM Press, 253–64 p. http://dx.doi.org/10.1128/9781555815615.ch15 ELSVER, K.F. AND LAMIN-SCOFIT, H.M. 2000. Encyclopedia of Microbiology. J.Lederberg (ed.), San Diego, California, Academic Press. HENTZER, M., RIEDEL, K., RASMUSSEN, T.B., HEYDORN, A., ANDERSEN, J.B., PARSEK, M.R., RICE, S.A., EBERL, L., MOLIN, S., HOBY, N., KJELLEBERG, S., GIVSKOV, M. 2002. Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. Microbiology, 148, 87–102. INOUE, T., SHINAKI, R. AND FUKUI, K. 2008. Inhibition of swimming motility of Pseudomonas aeruginosa by branched-chain fatty acids. FEMS Microbiology Letters, 281, 81–86. http://dx.doi.org/10.1111/j.1574-6968.2009.01909.x KRISTIANSEN, J.E. The antimicrobial activity of psychotropotherapeutic drugs and their stereo-isomeric analogues. [Ph.D. Thesis]. 1990. University of Copenhagen. Dan Med Bull, 37, 165–82.
2. The antimicrobial activity of non-


