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Structural differences between paroxetine and femoxetine responsible for differential inhibition of *Staphylococcus aureus* efflux pumps

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Abstract—In this study the chemical modification of paroxetine was employed to determine which structural differences between the paroxetine-like and femoxetine-like selective serotonin reuptake inhibitors is responsible for the differential potency of these agents in the inhibition of *Staphylococcus aureus* multidrug efflux pump systems.

Antimicrobial agent resistance is a major problem in the chemotherapeutic treatment of bacterial infections.[1] Recently, the action of membrane-based efflux pump systems has been shown to play a significant role in the development of multidrug resistance to antibiotics.[2] The development of efflux-mediated resistance to antibiotics generally occurs through the up-regulation of genes encoding transporters that efficiently expel the drug from the bacterial cell. As a direct resistance mechanism, the bacterial efflux pump affords subtherapeutic intracellular antibiotic concentrations by expelling substrates upon their entering the membrane or cytoplasm. Bacterial efflux pump systems also contribute indirectly to other types of resistance because exposure of bacteria to subtherapeutic drug concentrations promotes the selection and expression of higher-level adaptive resistance mechanisms, such as target mutations.[2,3] Constitutive expression of such pumps in some pathogenic organisms contributes significantly to their innate multidrug resistance phenotype.[2,4]

Due to the profound role of bacterial efflux pumps in the development of antimicrobial resistance much effort has been invested in the search for efflux pump inhibitors, which when combined with substrate antibiotics can restore antibiotic potency and reduce the occurrence of mutation-mediated resistance.[2b,5] The NorA protein was the first characterized pump to impart efflux-mediated multidrug resistance in *Staphylococcus aureus* (*S. aureus*).[6] Subsequently, a non-NorA-related multidrug efflux phenotype has been identified in *S. aureus*.[7] Inhibitors of the NorA efflux pump system in *S. aureus* have included variety of natural products and synthetic agents.[5a]

Recently, Kaatz et al. demonstrated that structural variants of certain phenylpiperidine selective serotonin reuptake inhibitors (P-SSRIs) inhibited the function of multidrug efflux pumps in *S. aureus* (Fig. 1).[8] The P-SSRIs were also shown to augment the potency of antimicrobial efflux pump substrates against strains of *S. aureus* possessing different efflux-related mechanisms and inhibited both NorA and non-NorA-related efflux phenotypes. For example, paroxetine (25 μg/mL) afforded an 8-fold reduction in the MIC of norfloxacin (50–6.3 μg/mL) against SA 1199B (NorA overproducing phenotype). This activity is similar to the 4-fold reduction in norfloxacin MIC produced by 20 μg/mL reserpine (a known bacterial efflux pump inhibitor). Paroxetine and reserpine each afforded an 8-fold reduction in the MIC of norfloxacin (12.5–1.6 μg/mL) against SA-K2068 (non-NorA phenotype).[8] This augmentation of antibiotic activity was shown to be a result of efflux inhibition; the P-SSRIs all have poor intrinsic antistaphylococcal activity. Evaluation of efflux pump inhibition by the P-SSRIs showed 'no' significant stereochemical requirement for pump inhibition, where

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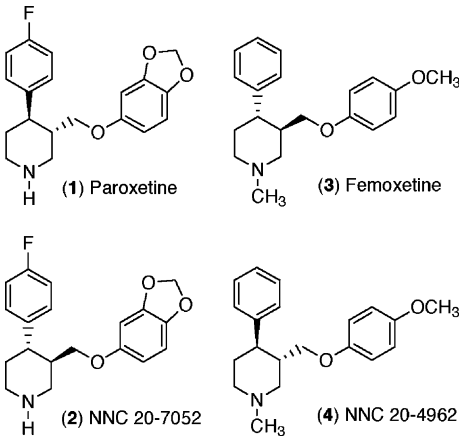
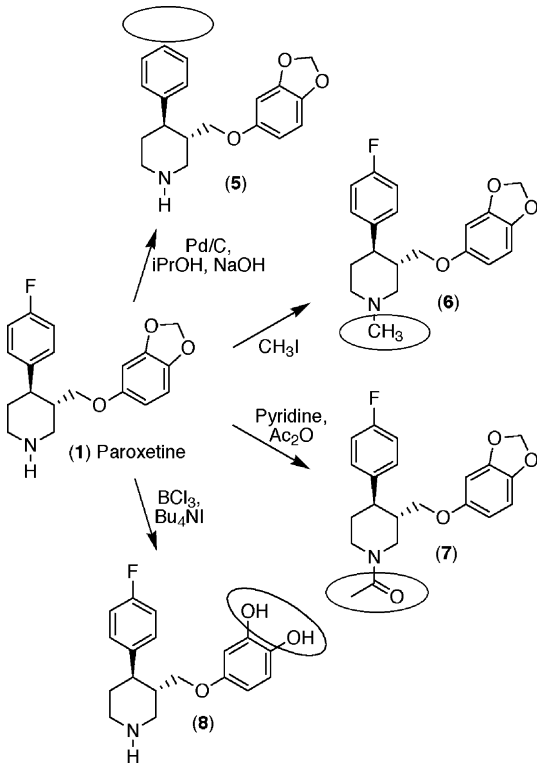


Figure 1. Structures of phenylpiperidine selective serotonin reuptake inhibitors that inhibit multidrug efflux pump systems in *S. aureus*.[8]

paroxetine and corresponding stereoisomer **2** displayed near equivalent activity, as did femoxetine and corresponding stereoisomer **4** (Fig. 1). However, paroxetine and **2** achieved 50% inhibition of the different *S. aureus* efflux pumps at 2- to 10-fold lower concentrations than femoxetine (**3**) and **4**.

In this study, we set out to determine which structural differences between the paroxetine-like and femoxetine-like P-SSRIs are responsible for differential inhibition of efflux pumps in *S. aureus*, and thus determine which peripheral structural features of the P-SSRIs are important for pump inhibition and which sites on the P-SSRI core structure are sensitive to substitution. These data will reveal sites on the P-SSRI core structure amenable to further chemical modification in an effort to increase potency of pump inhibition. The non-stereochemical structural differences between femoxetine-like and paroxetine-like P-SSRIs are: (1) the fluorine atom on the phenylpiperidine portion of paroxetine is absent in femoxetine, (2) the piperidine of paroxetine is unsubstituted while femoxetine is *N*-methylated, (3) the phenylether moiety of femoxetine bears a 4-methoxy-substituent versus the sesamol group (methylenedioxy substituent) of paroxetine (Fig. 1).

To this end, defluorinated paroxetine derivative **5** was prepared to evaluate the role of aryl fluoride substitution on the differential activity of paroxetine-like and femoxetine-like P-SSRIs (Scheme 1).[9] Preparation of the *N*-methyl paroxetine derivative **6** was similarly undertaken to determine if *N*-methylation of the piperidine ring in femoxetine is responsible for decreased pump inhibitory activity compared to paroxetine (Scheme 1).[10] While **6** was anticipated to establish the role of *N*-methylation in the differential activity of paroxetine and femoxetine, both the *N*-unsubstituted and *N*-methylated P-SSRIs are expected to be ionized under assay conditions. Thus, *N*-acetyl paroxetine derivative **7** was prepared to further evaluate the role of *N*-substitution on pump inhibitory activity and to potentially elucidate if there is a requirement for an ionizable piperidine ring (Scheme 1).[11] The phenylether substituent of paroxetine



Scheme 1. Synthesis of paroxetine derivatives employed in this study to evaluate the role of peripheral substituents on the differential pump inhibitory activity of paroxetine-like versus femoxetine-like P-SSRIs.[9–12]

and femoxetine are similarly substituted, but not equivalently, with the methylenedioxy and methoxy groups, respectively. To determine if this portion of the P-SSRI structure is sensitive to substituent effects and is important for pump inhibitory activity the methylenedioxy group of paroxetine was converted to catechol derivative **8**, affording an analog with a dramatic switch in the character of substituents on the phenylether ring.[12]

Relative potency of the P-SSRI efflux pump inhibitors was evaluated using the ethidium bromide (EtBr) efflux assay as previously reported (Fig. 2).[13] In this assay, increasing concentrations of the P-SSRIs and derivatives were evaluated for their inhibition of EtBr (pump substrate) efflux from two strains of *S. aureus* that possess either NorA (SA-1199B) or a non-NorA efflux pump (SA-K2068).

Efflux inhibition demonstrates that desfluoroparoxetine **5** and paroxetine inhibit EtBr efflux in both strains of *S. aureus* with near equivalent potency, indicating the aryl fluorine atom is not responsible for the difference in pump inhibitory activity between paroxetine and femoxetine (Fig. 2). Although the fluorine atom is small, the ability to make this subtle change on the phenylpiperidine structure indicates it may be possible to introduce structural changes on this ring toward identifying more potent pump inhibitors and toward preparing analogs devoid of CNS activities.

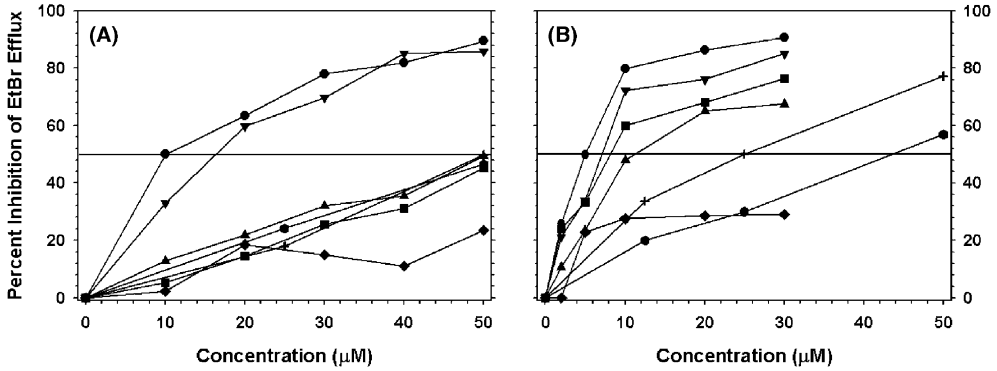


Figure 2. Effect of P-SSRIs and derivatives on EtBr efflux. (A) *S. aureus* 1199B, which constitutively over expresses *norA* and harbors a topoisomerase IV A subunit substitution (A116E); (B) *S. aureus* K2068, a strain demonstrating a non-NorA efflux-related multidrug resistance phenotype. The horizontal line indicates the concentrations necessary to inhibit efflux by 50%. ●, 1; ⬣, 3; +, 4; ▼, 5; ■, 6; ▲, 7; ◆, 8.

The *N*-methyl paroxetine derivative **6** displays less potent pump inhibition than paroxetine against both pump systems tested (Fig. 2). Most significant is that **6** inhibits 50% EtBr efflux against SA-1199B at approximately 50 μM, which is equivalent to the activity of femoxetine and **4** (Fig. 2). This indicates that the structural difference between paroxetine-like P-SSRIs and femoxetine-like P-SSRIs responsible for differential inhibition of efflux in SA-1199B is *N*-substitution of the piperidine ring. Furthermore, the *N*-acetyl derivative of paroxetine **7**, which can no longer carry a positive charge, also displays nearly identical inhibition of efflux to femoxetine, **4** and **6** in SA-1199B (Fig. 2). This observation that *N*-methylation and *N*-acetylation of paroxetine both produce femoxetine-like activity suggests either a putative requirement for the *N*-H group to form a positive binding contact in pump inhibition (e.g., hydrogen bond) or that *N*-substitution may be sterically less favorable.

The negative effect on pump inhibition observed for *N*-substituted paroxetine derivatives **6** and **7** in SA-1199B is also observed in the inhibition of efflux in SA-K2068, where *N*-methylation and *N*-acetylation afford approximately a 2-fold increase in concentration required to achieve 50% pump inhibition (Fig. 2). In this latter case however, the *N*-substituted paroxetine analogs still inhibit 50% efflux at 5- to 10-fold lower concentrations than **4** and femoxetine, respectively, indicating the subtle structural differences in the different substituents on the phenylether moiety are important to the inhibition of efflux in strain SA-K2068 but not strain SA-1199B. The overall importance of the phenylether substituents to pump inhibitory activity is exemplified by the clear decrease in pump inhibition observed for phenylether-modified paroxetine derivative **8** (Fig. 2). The effect of modifying the phenylether group on pump inhibition activity is more pronounced in the inhibition of efflux in SA-K2068 than SA-1199B, which is consistent with data discussed above where *N*-methyl paroxetine **6**, femoxetine and **4** showed similar inhibitory potency against efflux in SA-1199B while *N*-methyl paroxetine is a more potent inhibitor of efflux than femoxetine and **4** in SA-K2068. Thus, substituents on the phenylether portion of the P-SSRIs structure play an important role in potency of efflux inhibition. Further exploration of structure–function relationships around this portion of the P-SSRI structure is warranted toward identifying more potent pump inhibitors.

With respect to structural requirements of the P-SSRIs for CNS activity, the core 4-phenylpiperidine structure is common to many CNS active agents in addition to the P-SSRIs, likely due to similarities with the aryl-alkylamine pharmacophore of native neurotransmitters including serotonin, norepinephrine, and dopamine. The apparent requirement of an unsubstituted piperidine nitrogen for improved efflux pump inhibition shown here makes *N*-substitution an unlikely option to destroy CNS activity while increasing and/or broadening efflux pump inhibitory activity. However, remodeling the 4-phenylpiperidine core structure by changing the distance and/or orientation between the piperidine nitrogen and the phenyl ring may be one approach to identify improved efflux pump inhibitors that display decreased CNS activity (e.g., 3-phenylpiperidine derivatives, replacing the piperidine ring with alkyl amines of varied lengths to break the 4-phenylpiperidine pharmacophore, or incorporating substituents onto the piperidine ring). Perhaps most important is that P-SSRIs and other aryl-substituted alkylamine-based CNS agents accommodate only limited substitution of their aryl ring moieties, which is consistent with their requirement to bind sites on transporters that recognize the parent neurotransmitters. Thus, exploring a structurally diverse range of aromatic groups and aryl substituents for either of the aryl rings in the P-SSRI structure is likely a promising approach to initially identify derivatives having increased pump inhibitory activity and a concomitant decrease or elimination of CNS activity.

We have demonstrated that the disparity in potency of efflux pump inhibition between paroxetine-like and femoxetine-like P-SSRIs against NorA and non-NorA efflux pumps in *S. aureus* is primarily due to differing *N*-substitution of the piperidine ring. In addition, substituents on the phenylether moiety have been shown to play an important role in modulating potency of efflux inhibition, indicating further chemical modification of this ring may afford the opportunity to identify more

potent pump inhibitors. Continued structure–activity relationship studies to further characterize the pharmacophore of the P-SSRIs required for pump inhibition and to identify peripheral substitution of the phenylether and phenylpiperidine rings that improve potency and remove CNS activities are expected to reveal novel potent inhibitors of the efflux pump systems in *S. aureus*.

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10. *N*-Methylation of paroxetine to afford **6** was performed as follows. Methyl iodide (13.6 mg, 0.096 mmol) was added dropwise to paroxetine·HCl hemihydrate (30 mg, 0.08 mmol) and 55 mg potassium carbonate in 2 mL of dry acetonitrile. The reaction mixture was then heated to reflux for 5 h, cooled, the solution filtered and filtrate evaporated to dryness. Pure **6** was isolated as a hygroscopic white solid in 92% yield employing HPLC separation and analysis as described for **5**. ^{1}H NMR ($CDCl_3$) δ 1.82–1.92 (m, 2H), 2.04–2.14 (m, 2H), 2.19–2.29 (m, 1H), 2.38 (s, 3H), 2.44 (m, 1H), 2.97 (d, 1H), 3.21 (d, 1H), 3.44 (m, 1H), 3.59 (m, 1H), 5.87 (s, 2H), 6.17 (m, 1H), 6.38 (s, 1H), 6.65 (d, 1H), 6.96 (m, 1H), 7.19 (m, 1H). ^{13}C NMR ($CDCl_3$) δ 34.20, 42.01, 43.43, 46.36, 56.11, 59.44, 69.47, 97.96, 101.06, 105.57, 107.83, 115.28, 115.56, 128.77, 128.87, 139.50, 141.57, 148.14, 154.35, 159.07, 163.24. MS, ESI, calcd ($M+H^+$) 344, found ($M+H^+$) 344.
11. Acetic anhydride (0.1 mL) was added dropwise to a stirred solution of paroxetine·HCl hemihydrate (30 mg, 0.08 mmol) and catalytic DMAP in 2 mL dry pyridine under N_2. The mixture was stirred at room temperature for 2 h then evaporated to dryness. Pure **7** was isolated in 95% yield as a white solid employing HPLC separation as described for **5**. ^{1}H NMR ($CDCl_3$) δ 1.68–2.04 (m, 3H), 2.20 (s, 3H), 2.62–2.82 (m, 2H), 3.05–3.25 (m, 1H), 3.44–3.51 (m, 1H), 3.62–3.68 (m, 1H), 3.90–4.19 (m, 1H), 4.75–4.95 (m, 1H), 5.87 (s, 2H), 6.17 (m, 1H), 6.38 (s, 1H), 6.65 (d, 1H), 6.94 (m, 1H), 7.17 (m, 1H). MS, EI, calcd 371, found 371.
12. Cleavage of the methylenedioxy group utilized the method of Brooks et al.: Brooks, P. R.; Wirtz, M. C.; Vetelino, M. G.; Rescek, D. M.; Woodworth, G. F.; Morgan, B. P.; Coe, J. W. *J. Org. Chem.* **1999**, *64*, 9719–9721. Paroxetine·HCl hemihydrate (20 mg, 0.053 mmol) and *n*-butyl ammonium iodide (49 mg, 0.132 mmol) were stirred in 2 mL dry CH_2Cl_2 at −78 °C under N_2. BCl_3 (0.132 mL of a 1 M solution in CH_2Cl_2) was added dropwise and the mixture stirred for 2 h. The reaction was quenched by addition of ice water and stirred at room temperature for 30 min. The mixture was extracted with CH_2Cl_2, the organic layer dried over Na_2SO_4, filtered, and evaporated to dryness. Pure **8** was isolated in 70% yield as a hygroscopic solid employing HPLC separation as described for **5**. ^{1}H NMR ($CDCl_3$) δ 1.43 (m, 2H), 1.82 (m, 2H), 2.20 (m, 1H), 2.71 (m, 1H), 2.82–2.92 (m, 2H), 3.28–3.42 (m, 4H), 5.97 (m, 1H), 6.24 (d, 1H), 6.56 (d, 1H), 6.76–6.86 (m, 2H), 6.96–7.04 (m, 2H). MS EI, calcd 317, found 317.
13. Kaatz, G. W.; Seo, S. M.; O'Brien, L.; Wahiduzzaman, M.; Foster, T. J. *Antimicrob. Agents Chemother.* **2000**, *44*, 1404–1406. Briefly, cells were grown in Mueller–Hinton broth (MHB; Difco, Detroit, MI) to late log phase (OD_{660} 0.8), at which time 25 μM ethidium bromide (EtBr) and

100 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were added to load cells with EtBr. After 20 min incubation at room temperature the OD_{660} was adjusted to 0.4 using fresh MHB containing EtBr and CCCP. Cells in 1 mL of this dilution were pelleted and then resuspended in fresh MHB with or without various concentrations of inhibitors. The fluorescence of the suspension was monitored continuously for 5 min (excitation and emission wavelengths, 530 and 600 nm, respectively). Percent inhibition of efflux was calculated by determining the difference in total efflux versus that observed in the presence of an inhibitor.