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3D-QSAR study of bis-azaaromatic quaternary ammonium analogs at the blood–brain barrier choline transporter

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Abstract—Previously, we have developed 3D-QSAR models of the blood–brain barrier (BBB) choline transporter, a transport system that may have utility as a vector for central nervous system drug delivery. In this study, we extended the model by evaluating five bis-azaaromatic quaternary ammonium compounds for their affinity for the choline binding site on the BBB-choline transporter. The compounds, and their affinities for the transporter, were then incorporated into our existing molecular model, in order to update our knowledge on the molecular recognition factors associated with interaction of ligands at the choline binding site. The current compounds are structurally related to previous substrates that we have evaluated, but offer additional three dimensional aspects compared to the series of compounds previously utilized to define the original models. The compounds showed good affinity for the BBB-choline transporter, exhibiting inhibition constants ranging from 10 to 68 μM, as determined by the in situ rat brain perfusion method. Comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA) methods were used to build the new 3D QSAR models. When the new bis-azaaromatic quaternary ammonium compounds were included in the model, the best cross-validated CoMFA q^2 was found to be 0.536 and the non-cross-validated r^2 was 0.818. CoMSIA hydrophobic cross-validated q^2 was 0.506 and the non-cross-validated r^2 was 0.804. This new model was able to better predict BBB-choline transporter affinity of hemicholinium-3 (predicted 65 μM, actual 54 μM), when compared to an earlier model (predicted 316 μM).

1. Introduction

The blood–brain barrier (BBB) is a major obstacle for the entry of drugs into the central nervous system (CNS). About 98% of potential CNS-active drugs are excluded from the brain by the BBB.[1] A viable alternative option to structural modification of a drug to facilitate brain entry is to utilize native nutrient transporters located at the barrier interface to bind to, and transport such drugs into the CNS.[2] Thus, drugs can be designed to have affinity for a BBB transporter, enabling relatively polar, water-soluble CNS-active drugs to enter the brain from the periphery.[1] This method has been successfully employed in early studies for compounds such as D,L-2-amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (D,L-NAM), a treatment for brain tumors[3,4] that was modified to use the BBB large neutral amino acid transport system. Another example is the neuronal nicotinic receptor antagonist *N*-*n*-octylnicotinium iodide (NONI, Fig. 1), which has been shown to bind the BBB-choline transporter with an affinity comparable ($K_i \sim 49$ μM) to that of choline ($K_m \sim 45$ μM).[5]

Furthermore, NONI was demonstrated to be transported by the BBB-choline transporter, which confirmed that this transport system has utility as a brain delivery vector. In the absence of an X-ray crystal structure for the human BBB-choline transporter,[6] we have previously developed a comparative molecular field analysis (CoMFA) model, to yield further insight into the requirements for ligand-transporter binding.[7,8] The results of this study generated the model for binding

Keywords: Blood–brain barrier; Choline; Brain drug delivery; Transport; Molecular modeling.
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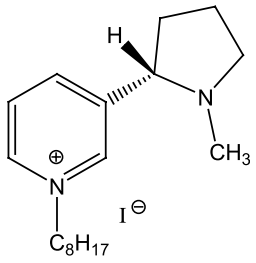


NONI

Figure 1. Structure of NONI, a quaternary ammonium compound that has affinity for the BBB-choline transporter. NONI has been shown to be taken up into the brain by the BBB-choline transporter, demonstrating the use of this transporter as a drug delivery vector.

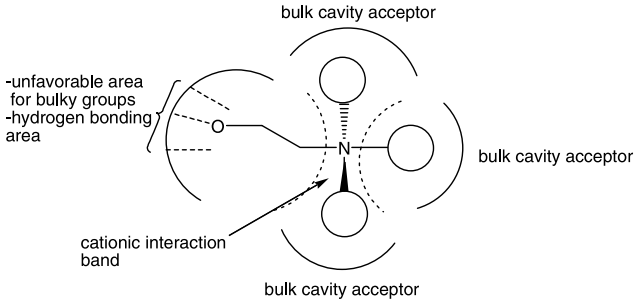
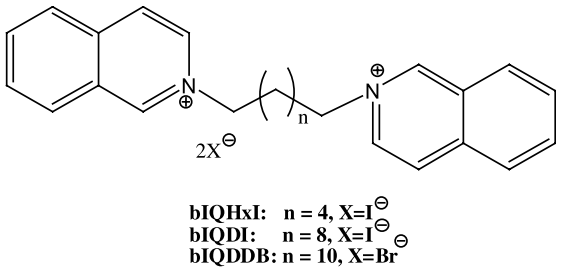
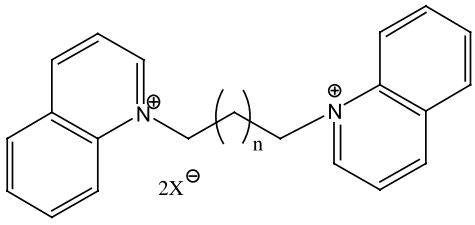


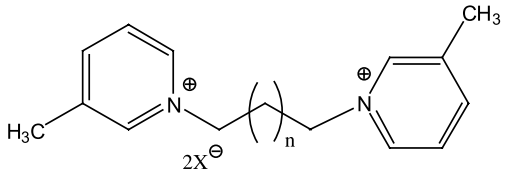
Figure 2. Theoretical rendering of the active binding site of the BBB choline transporter.[2,7,8]



bIQHxI: n = 4, X=I
bIQDI: n = 8, X=I
bIQDDB: n = 10, X=Br



bQHxI: n = 4, X=I



bPiDDB: n = 10, X=Br

Figure 3. Structures of bis-azaaromatic quaternary ammonium compounds used in this study.

interaction illustrated in Figure 2 below.[2,7,8] Structure–activity relationships suggested the need for a quaternary ammonium group, a free hydroxyl group, and an intramolecular distance between the quaternary ammonium nitrogen and the hydroxyl group of ~3.3 Å. Surprisingly, it was also shown that bis-quaternary ammonium compounds lacking a hydroxyl group also inhibit choline uptake into brain synaptosomes.[9]

In this report, we discuss the exploitation of the BBB-choline transporter as a drug delivery vector for bis-compounds containing two charged quaternary ammonium moieties in their molecular structure.[5,7,8] The BBB-choline transporter is responsible for transporting choline, a charged cation, into the CNS, where it acts as a precursor for the neurotransmitter acetylcholine and also as an essential component of membrane phospholipids such as phosphatidylcholine.[10]

Novel *N*-*n*-alkylnicotinium analogs have been demonstrated to modulate the dopaminergic system[11,12] by inhibiting nicotine-evoked dopamine overflow from rat striatal slices. In addition, these compounds are reported to cross the BBB by acting as substrates for the BBB-choline transporter.[5] A series of bis-azaaromatic quaternary ammonium compounds (Fig. 3) have recently been synthesized and evaluated for CNS activity.[13–15] These compounds were shown to be ligands for neuronal nicotinic receptors,[13] with suggested possible utility in Alzheimer's and Parkinson's disease, and smoking cessation therapies.[13] Because the pharmacological profiles of some of these compounds are similar to that of NONI, they are of interest in expanding our understanding of the nature of the pharmacophoric requirements of the BBB-choline transporter, since the charged quaternary ammonium moiety largely prevents passive uptake into the brain due to the BBB's selective permeability.

In the present study, we supplemented and refined the predictive utility of our earlier BBB-choline transporter 3D-QSAR model by adding the novel bis-azaaromatic quaternary ammonium compounds to the equation. We propose that information gleaned from this study provides a better understanding of how the rational incorporation of specific structural moieties into CNS-active drug molecules may also allow them to act as substrates for the BBB-choline transporter. Additionally, the incorporation of these bis-azaaromatic quaternary ammonium compounds into our model may provide us with a better understanding of why these compounds have affinity for the BBB-choline transporter, even though they lack a free hydroxyl group, which was previously thought to be necessary for high affinity.[9]

2. Methods

2.1. Chemistry

NONI was prepared as previously described.[11] The bis-azaaromatic quaternary ammonium compounds, bIQHxI, bIQDI, bIQDDB, bQHxI, and bPiDDB, were pre-

pared by the procedures described by Ayers et al.[13] Briefly, the compounds were synthesized by reacting an appropriate azaaromatic compound with a variety of diiodo- or dibromoalkanes for 24 h at 65 °C. Synthesis, structure determination using ^{1}H and ^{13}C NMR spectroscopy and mass spectroscopy, and purity determination by elemental analysis were carried out as previously described.[13] The structures of all compounds were consistent with the spectroscopic data.

2.2. In situ brain perfusion technique

Brain uptake of [^{3}H]-choline was assessed using a modified in situ rat brain perfusion technique.[16–18] Briefly, for this study, short perfusions (60 s) were used to determine [^{3}H]-choline brain uptake. Once uptake values were estimated, subsequent experiments evaluated the inhibition of [^{3}H]-choline brain uptake by 250 μM concentrations of each of the test compounds. This concentration is our standard starting point for compound screening, because it represents approximately five times the k_m of choline for the transporter.[10]

Male Fischer-344 rats (220–250 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center. Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal) by a PE-60 catheter containing heparinized saline (100 U/ml), which was inserted into the left common carotid artery after ipsilateral external carotid, occipital, and common carotid artery ligation (the latter ligation was caudal to the catheter implantation). The pterygopalatine artery was patent during these experiments.[10] Rat body temperature was evaluated and maintained at 37 °C by a feedback-controlled heating pad (YSI Indicating Controller, Yellow Springs, Ohio). The left common carotid artery catheter was connected to a syringe, which contained physiologic buffered perfusion fluid (containing [in mM]: NaCl 128, $NaPO_3$ 2.4, $NaHCO_3$ 29.0, KCl 4.2, CaCl 1.5, $MgCl_2$ 0.9, and D-glucose 9) with 1 μCi/ml [^{3}H]-choline and 0.3 μCi/ml [^{14}C]-sucrose (to determine vascular volume). Perfusion fluid was filtered, warmed to 37 °C, and gassed with 95% O_2 and 5% CO_2. Just before perfusion, pH and osmolarity of this solution were ≈7.35 and 290 mOsm, respectively. Perfusion fluid was infused into the left carotid artery at 10 ml/min for 60 s using a Harvard Apparatus infusion pump (South Natick, MA). This perfusion rate maintains a carotid artery pressure of ~120 mmHg.[10,16] After 60 s perfusion with [^{3}H]-choline (in the absence and presence of the unlabeled compound of interest), perfusion fluid was switched to contain tracer-free perfusion fluid for 15 s to wash out residual [^{3}H]-choline loosely bound to the cerebrovasculature.[18]

Inhibition of [^{3}H]-choline brain uptake was ascertained by addition of the appropriate unlabeled bis-azaaromatic quaternary ammonium analog into the perfusion fluid. To determine K_i values, compounds were evaluated at concentrations of 250 μM, as described previously[19] and briefly detailed below.

Rats were decapitated and brain samples obtained as described previously.[16] Briefly, the brain was removed from the skull, the arachnoid membrane and meningeal vessels were removed, and the perfused cerebral hemisphere dissected on ice. Brain regions were placed in scintillation vials and weighed. Two 50 μl aliquots of the perfusion fluid were also transferred to the individual scintillation vials and weighed. Brain and perfusion fluid samples were digested overnight at 50 °C in 1 ml piperidine (1 M) solution. Fisher chemical scintillation cocktail (10 ml; Beckman, Fullerton, CA) was added to each vial and the tracer contents assessed by dual-label liquid scintillation counting, and corrected for quench, background, and efficiency.

Brain [^{3}H]-choline uptake was determined by a single time point blood-to-brain transfer coefficient (K_{in}) calculation as previously described,[16,20] using the following relationship:

$$K_{in} = [C_{tot} - V_v C_{pf}]/(C_{pf}/T) \quad (1)$$

where $C_{tot} = C_{br} + C_{vas}$ is the sum amount of choline contained in the perfusate in the blood–brain vessels (C_{vas}) and the amount of choline that penetrated into the brain (C_{br}). Cerebral vascular volume (V_v) and perfusion flow rate were estimated in separate experiments as previously described.[2] C_{pf} is the tracer choline perfusion fluid concentration, and T is the net perfusion time assumed to be under linear uptake conditions.

Apparent cerebrovascular permeability surface-area products (PA) were determined using the following relationship:

$$PA = F \ln(1 - k_{in}/F) \quad (2)$$

In this relationship, cerebral blood flow (F) was assessed for the brain region of interest from uptake of [^{14}C]-diazepam, as described earlier.[16]

Apparent inhibition constants (K_i) for bis-azaaromatic quaternary ammonium compounds were determined as previously described,[19] from the equation:

$$[(PA_o - K_D)/(PA_i - K_D)] = 1 + C_i/K_i \quad (3)$$

Competition kinetics were assumed, and from this relationship, PA_o represents the [^{3}H]-choline PA in the absence of competitor. PA_i represents the [^{3}H]-choline PA in the presence of the inhibitor and C_i represents the perfusate concentration of inhibitor. Apparent K_i is defined as the inhibitor concentration that reduces saturable brain [^{3}H]-choline influx by 50% at tracer choline concentration ($C_{pf} \ll K_m$), in the absence of competing compounds. We were able to demonstrate competition at the BBB-choline transporter over a 0.25–12.5 μM concentration range of hemicholinium-3, the defining substrate for choline transport.[18] This range has previously demonstrated that competition kinetics occur at the BBB-choline transporter and provides the basis for evaluating K_i values for the inhibitors.[18]Additionally,

previous studies indicate that monocations such as sodium have little effect on choline transport.[18] Furthermore, while the concentrations of the compounds utilized in the present study were used solely to evaluate their effects on BBB choline uptake, it should be pointed out that therapeutic concentrations of the same compounds would likely be much lower, due to their effects on brain dopamine systems.[15]

3. Molecular modeling

Molecular modeling was accomplished as described previously.[8] Briefly, molecules were built using SYBYL 6.91 (Tripos Inc., St. Louis, MO) molecular modeling software on a Silicon Graphics Octane computer. All molecules were energy minimized for CoMFA and CoMSIA analysis by iteratively searching for lowest energy conformations by rotating bonds in 10° increments followed by energy minimization. All compounds were aligned manually using the FIT ATOMS function in SYBYL, by superimposing the cationic N-atom, the α-carbon adjacent to the nitrogen atom and the hydroxyl group. CoMFA analyses were carried out using a standard SYBYL/QSAR routine. CoMFA grid spacing was 2.0 Å in the *X*, *Y*, and *Z* directions, and grid regions were automatically generated by the CoMFA routine to encompass all molecules with an extension of 4.0 Å in each direction. An sp^3 carbon for measuring steric interactions and a point charge of +1.0 to explore electrostatics were used as probes to generate the interaction energies at each lattice point, with a default energy cutoff at 30 kcal/mol. Partial least squares (PLS, cross-validated and non-cross-validated) regression analyses were performed from the resulting field. With cross-validation, one compound is taken out of the group, and the remaining compounds are then used to generate a new model. This model is then used to predict the biological activity of the deleted compound. This value (q^2) differs from the normal regression value (r^2), in that the q^2 value can be negative. Column filtering (2.0 kcal/mol) was also investigated to see if such an approximation would result in improved q^2 values. In PLS, each compound is described by different dimensions, denoted principal components, and PLS attempts to then identify components that are relevant in interactions between the biological parameter (e.g., $\log K_i$) and molecular descriptors (e.g., steric or hydrophobic fields). The number of components was chosen such that the smallest standard error of prediction was achieved, where these components describe the degree of complexity of the model. Usually, the minimum number is chosen, since adding more components may lead to over-fitting of the data, which results in low predictability. These relative contributions are represented as a 3D coefficient map illustrating the favored effects [80%; steric (in green) and electrostatic (in blue)] and the unfavorable effects [20%; steric (in yellow) and electrostatic effects (in red)]. The green map areas indicate where sterically bulky groups may enhance binding affinity. Blue colored regions indicate where electronegative groups will enhance binding affinity. $\text{Log}\,K_i$ values were used as the biological descriptor in the SYBYL spreadsheets.

3.1. Radiochemicals

[^{3}H]-Choline (79.2 Ci/mmol, >98% purity) and [^{14}C]-sucrose (4.75 mCi/mmol) were obtained from Dupont-New England Nuclear (Boston, MA). Prior to each perfusion, [^{3}H]-choline was thoroughly dried before being dissolved in perfusion buffer to ensure that volatile tritium contaminants (such as [^{3}H]–H_2O) were not present.

4. Results and discussion

An established approach for augmenting brain drug delivery is to utilize native BBB saturable nutrient transporters, such as has been demonstrated with the BBB-choline transporter.[2,5] In this report, we evaluated how the inclusion of a new series of bis-azaaromatic quaternary ammonium compounds would influence our earlier 3D-QSAR model of the BBB-choline transporter. This study was initiated to further understand how this transport system could serve as a vector for CNS drug delivery. Additionally, these bis-azaaromatic quaternary ammonium compounds may provide insight as to why they show high affinity for the BBB-choline transporter, even though they lack a free hydroxyl group in their structure. The bis-azaaromatic quaternary ammonium compounds were evaluated for their ability to block [^{3}H]-choline uptake into brain via the BBB-choline transporter, utilizing the in situ brain perfusion technique.[10] Inhibition constants ($\log K_i$) estimated for these compounds are given in Table 1.

CoMFA and CoMSIA techniques were employed to gain insight into the structural requirements for these compounds to bind to the transporter, as well as to derive extended predictive models for future drug design. Results of CoMFA and CoMSIA analyses are shown in Tables 2 and 3, respectively. Two models are presented in this study: (1) our current model (Model 1)[8] and (2) a new model with the bis-azaaromatic quaternary ammonium compounds included (Table 2). For the first model,[8] a cross-validated q^2 of 0.406 was estimated, which improved when column filtering of 2.0 kcal/mol was applied, to give a final q^2 of 0.408 (Table 2). A non-cross-validated r^2 of 0.943 was determined for this model. Steric and electrostatic contributions were calculated to be 72% and 28%, respectively, supporting our earlier findings suggesting that the steric properties of the cationic compounds are of paramount importance in transporter binding.

Table 1. Inhibition of brain [^{3}H]-choline uptake, $\log K_i$ (μM) ± standard error of the mean (SEM) values obtained from in situ brain perfusion studies[9] for bis-azaaromatic quaternary ammonium compounds used in this study

Compound	$\log K_i$ (μM) ± SEM
bPiDDB	1.57 ± 0.48
bQHxI	1.83 ± 1.18
bIQHxI	1.46 ± 0.70
bIQDI	0.98 ± 0.26
bIQDDB	1.35 ± 0.18

Table 2. Summary of CoMFA partial least square (PLS) analysis of the original model (Model 1) and the new model with the bis-azaaromatic quaternary ammonium compounds (Model 2) included in the training sets

Model	q^2	S.E.P.	Components	r^2	S.E.E.	Steric	Electrostatic
1	0.408[a]	0.875	2	0.943	0.290	0.717	0.283
2	0.536[a]	0.881	2	0.818	0.130	0.637	0.363

[a] Column filtering of 2 kcal/mol; S.E.P. = standard error of prediction; S.E.E. = standard error of estimate.

Table 3. Summary of CoMSIA (hydrophobic) PLS analysis of the original model (Model 1) and the new model with the bis-azaaromatic quaternary ammonium compounds (Model 2) included in the training sets

Model	q^2	S.E.P.	Components	R^2	S.E.E.
1	0.433	0.864	1	0.883	0.416
2	0.506	0.819	3	0.804	0.531

S.E.P. = standard error of prediction; S.E.E. = standard error of estimate.

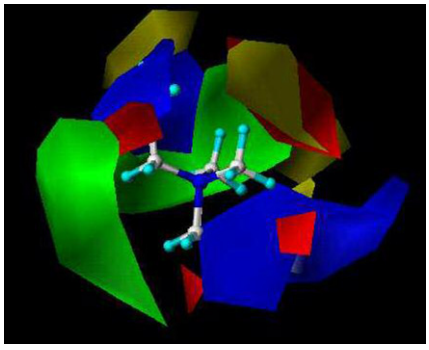


Figure 4. Contour maps of the CoMFA analysis for the original training set without inclusion of the bis-azaaromatic quaternary ammonium compounds (Model 1). Green areas indicate regions where bulky substituents can be accommodated in a sterically favorable way, and yellow, in an unfavorable way. Electrostatic fields are shown where blue areas would favorably accommodate cationic groups. Cationic interactions that would be unfavorable are shown in the red areas. The docking of choline into the binding site is shown in the

Figures 4–6 show the resulting CoMFA contour plots representing the new model (Model 2) with the bis-aza-

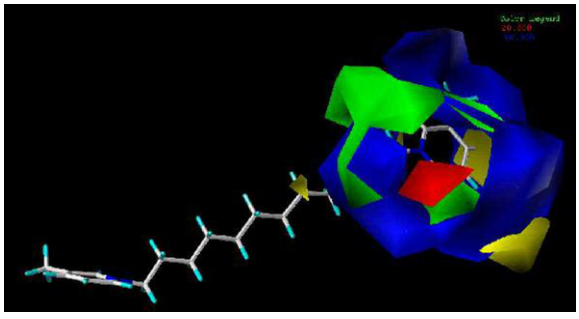


Figure 6. Contour maps representing the new CoMFA analysis (Model 2). The bis-azaaromatic quaternary ammonium compound bPiDDB (one of the new compounds) is shown docked into the binding site.

aromatic quaternary ammonium compounds included, and the original model (Model 1) for comparison. The original CoMFA model was used both for comparison, and to investigate its robustness to predict a 3D-QSAR for new, chemically diverse compounds. Most 3D-QSAR training (and test) sets contain compounds with a similar substructure, with side chain replacement as the major variant among the compounds. The structural models investigated herein display a variety of structural features in which even the substructures differ considerably. These range from ring structures to alkyl compounds.

The green contours in Figures 4–6 represent high steric tolerance regions (80% contribution) while yellow contours represent regions of unfavorable steric interaction (20% contribution). Electrostatic contours are shown in blue and red. Blue contours represent regions where positively charged groups will enhance activity

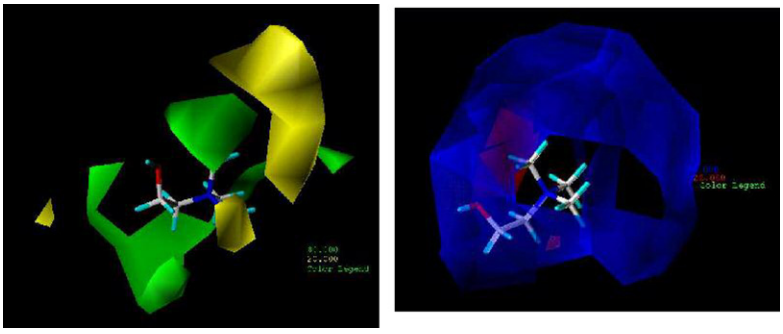


Figure 5. Contour maps of the new CoMFA analysis (Model 2), which include the bis-azaaromatic quaternary ammonium compounds in the training set. The steric and electrostatic fields are shown separated. Green areas indicate regions where bulky substituents can be accommodated in a sterically favorable way, and yellow, in an unfavorable way. Electrostatic fields are shown where blue areas would favorably accommodate cationic groups. Cationic interactions that would be unfavorable are shown in the red areas. The docking of choline into the binding site is shown in the above model.

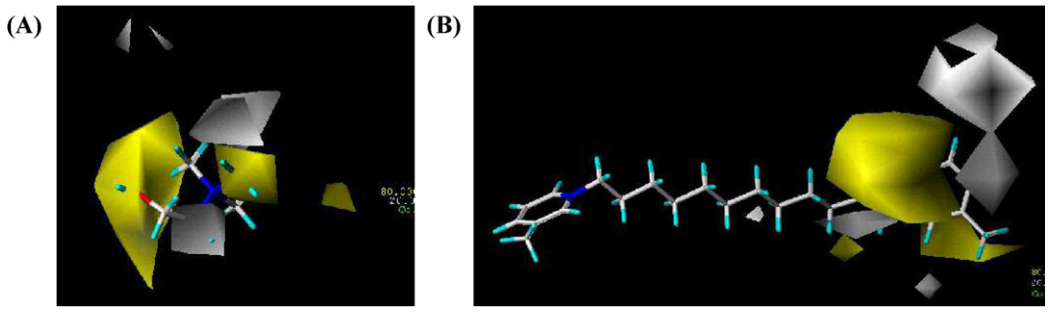


Figure 7. Contour maps of the CoMSIA hydrophobic fields with (A) the original model (Model 1), and (B) the new model (Model 2) with the bis-azaaromatic quaternary ammonium compounds included in the training set. Areas that favor hydrophobic interactions are indicated by yellow, and areas that are unfavorable for hydrophobic interactions are indicated in white. Choline docked into the binding site is illustrated in the left panel (A) and bPiDDB, a representative of the bis-azaaromatic quaternary ammonium compounds, is shown docked into the binding site in the right panel (B).

(80% contribution), whereas red regions denote where negatively charged groups will enhance activity (20% contribution). Green areas surround the alkyl chain segment of choline, as well as an area near the *N*-methyl groups (Fig. 5). The latter areas indicate where bulky groups would sterically complement the interaction between a ligand and the choline transporter binding site. The same green 'band' is also seen in Figure 5, suggesting a commonality between these two models. Figures 4 and 5 show a large ('favorable') blue area surrounding the cationic nitrogen, as would be expected for a cationic transporter, and indicates a region where positively charged substituents would be predicted to improve affinity. Figure 6 shows the new CoMFA model (Model 2) with bPiDDB docked into the binding site.

Figure 7 shows the results of CoMSIA hydrophobic field analyses. Favorable hydrophobic fields are indicated by yellow contours and unfavorable regions are indicated by white contours. The original model (Model 1, Fig. 7A)[8] shows primarily two areas surrounding the cationic quaternary ammonium moiety of choline. The white areas show where more hydrophilic substituents in the structure of the compounds would likely increase affinity for the choline transporter, while yellow areas show where the addition of hydrophobic substituents may increase affinity for the binding site. The new model (Model 2, Fig. 7B) with the bis-azaaromatic quaternary ammonium compounds included in the modeling, shows similar contour maps with the yellow regions surrounding the cationic quaternary ammonium group in bPiDDB, which is also shown docked into the binding site (Fig. 7B).

It is important to note that the bis-quaternary ammonium compounds exhibit a high affinity for the choline transporter, even though they lack a hydrogen bonding moiety in their structure. When the new bis-quaternary ammonium compounds were docked into the Model 1 binding site, we were unable to adequately predict the affinities of these compounds. The bis-azaaromatic quaternary ammonium compounds take up molecular space not encountered in the training set used to create the original CoMFA model. This was evident when our original model was used to predict the $\log K_i$ for these bis-compounds. No correlation could be found between predicted and actual brain [^{3}H]-choline uptake inhibition (i.e., $\log K_i$s, predicted $\log K_i > 4$) for the new bis-azaaromatic quaternary ammonium compounds.

The bis-azaaromatic quaternary ammonium compounds differ substantially from the training set compounds used in the previous study to create Model 1. First, the compounds used for creating Model 2 contain two positively charged centers, as opposed to one positively charged center in the molecules used in the original training set for Model 1. Second, the bis-quaternary ammonium compounds are devoid of the hydroxyl group present in the choline molecule, which is prominent in a large proportion of the compounds in the original training set for Model 1. The hydroxyl group is thought to contribute to the high affinity of molecules for the choline binding site in the Model 1 approximation (Fig. 2).

Two possible explanations could account for these observations. First, one of the cationic moieties may initially bind to the anionic site on the BBB-choline transporter, anchoring the molecule to the transporter; subsequently, the second cationic moiety may then bind to a second anionic site on the transporter, which is different from the hydrogen bonding site in the original model. Because of the high degree of flexibility and the length of the tethered *n*-alkyl chain in the bis-azaaromatic compounds, it is likely that the anchored molecule can adopt a large number of conformations. For example, two extreme conformations of the bis-azaaromatic compounds that may be involved in binding to the transporter are: a fully extended conformation, where the two cationic moieties are furthest apart and interact with two separate anionic binding sites; or alternatively, a folded or hairpin conformation where both cationic moieties interact with one anionic binding site, or with two closely juxtaposed binding sites on the transporter. Of course, other possible conformations of these flexible molecules may also be recognized by the transporter binding site. The *n*-alkyl linker may serve a secondary role in the binding process by interacting with hydrophobic residues adjacent to the anionic binding site(s) lining the transporter cavity. Secondly, it is possible that the bis-azaaromatic compounds interact with a dimeric complex of the choline transporter by spanning the dis-

tance between two adjacent anionic sites on individual transporter molecules (i.e, the bis-azaaromatic compounds may bind in an intermolecular manner with the transporter dimer). In this respect, it has been proposed that transporters, such as the neuronal dopamine transporter, consist of several transmembrane units.[21] Also, G-protein-coupled receptors are known to exist as functional dimeric units,[22,23] with closely associated binding pockets between the different receptor subunits.

Both models could explain why bis-quaternary ammonium compounds have affinity for the BBB-choline transporter, even though they do not possess a free hydroxyl group. Our proposed syntheses of rigid analogs that conformationally restrict these molecules into folded, extended or other unique structures will help determine the active conformation of these flexible bis-azaaromatic aromatic quaternary ammonium compounds.

The second cationic quaternary ammonium group tethered to the first seems to compensate for the loss of the hydrogen bond interaction proposed for ligands with a free hydroxyl group.[9] To address this concept in our molecular modeling approach, we carried out the following: we docked only one of the cationic moieties of the bis-azaaromatic quaternary ammonium structure into the original CoMFA model. Accordingly, we truncated the bis-azaaromatic quaternary ammonium compounds, revealing three distinct substructures (Fig. 8).

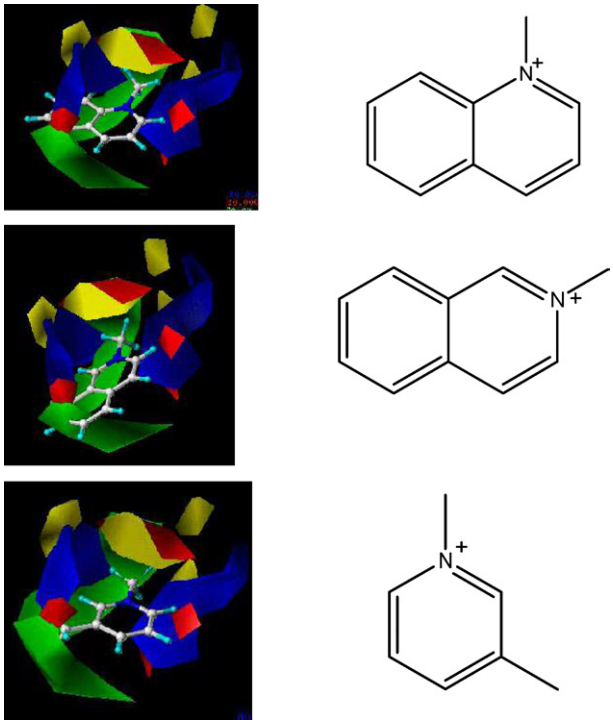


Figure 8. Contour maps of the original CoMFA analysis (Model 1), without the bis-azaaromatic quaternary ammonium compounds in the training set. The three truncated cationic moieties derived from the bis-azaaromatic quaternary ammonium compounds are shown docked into the original CoMFA model.

As seen in Figure 8, these cationic moieties were aligned with choline in such a way as to orient the *n*-alkyl linker toward a green, sterically favored area in the first CoMFA model (Model 1), and with the cationic quaternary ammonium groups overlapping. The cationic moieties appear to fit comfortably inside the CoMFA model in this configuration. As expected, the predicted $\log K_i$s were not in agreement with experimental values ($\log K_i \approx 3$), likely due to the absence of the hydroxyl group, which appears to be important for high affinity binding of most, but not all, BBB-choline transporter ligands.[2] This confirmed our speculation that the tethered second cationic group is required for high affinity interaction or binding at the BBB-choline transporter (as is seen with these bis-quaternary ammonium compounds), when compared to choline. Thus, in the bis-compounds, the incorporation of two cationic moieties into the molecule may compensate for the lack of a hydroxyl group thought to be necessary for hydrogen bonding at the choline binding site (see Fig. 2).

Because our original model (Model 1) failed to adequately predict the affinity of the bis-quaternary ammonium compounds, we evaluated whether incorporation of these compounds into the first model would improve our 3D QSAR descriptors. The new model's (Model 2, Table 2) cross-validated q^2-value was lower than that of the original model with an estimated q^2 value of 0.374. With column filtering set at 2.0 kcal/mol, the new model improved further to yield a final model with a q^2 of 0.536 and a non-cross-validated r^2 of 0.881.

To test the second model's predictive capability, and as proof of concept, we searched for compounds that incorporate two quaternary ammonium groupings joined by an *n*-alkyl chain linker in the bis-azaaromatic compounds, leading us to evaluate hemicholinium-3 and hemicholinium-15 (Fig. 9).

Hemicholinium-3 is a bis-quaternary ammonium compound similar to the bis-azaaromatic compounds, but it also contains additional hydroxyl groups in its structure. Hemicholinium-3 is the defining substrate for choline transport systems.[2] Hemicholinium-15 is the monomeric analog of hemicholinium-3, and was a component of the training set in both CoMFA models described above. The original CoMFA model predicted hemicholinium-3 to have a K_i of 316 μM, which changed to 63 μM, when the new CoMFA model was utilized. The experimental K_i of hemicholinium-3 is 54 μM.[2] Hemicholinium-15 binds with much lower affinity than hemicholinium-3, suggesting that the binding of two cationic quaternary ammonium groups results in higher affinity and potentially greater transport than binding of just one cationic group. This also indicated that, by incorporating bis-compounds in our previous model, it is possible to use this new model for future studies that will attempt to predict the binding properties of bis-quaternary ammonium compounds.

The results of this study suggest that determining the maximum size of the ligand that can be accommodated by the BBB-choline transporter will aid in the design of

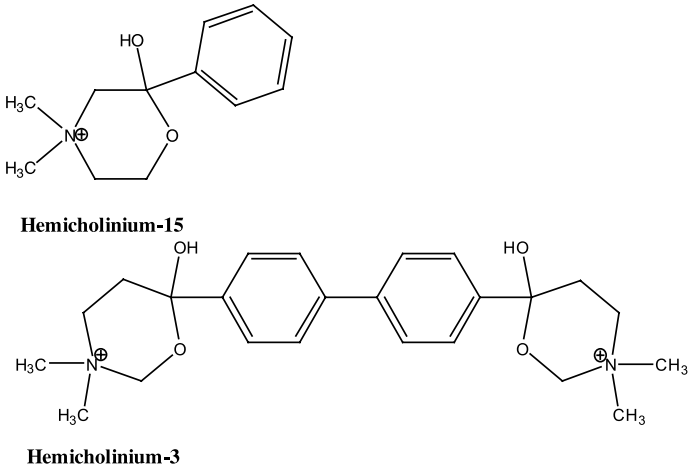


Figure 9. Structures of hemicholinium-3, a bis-quaternary ammonium compound, and its monomer, hemicholinium-15.

pro-drugs or co-drugs of molecules that poorly penetrate the BBB. Thus, tethering the poorly permeable drug to a high affinity ligand may allow shuttling of the drug into the CNS via the BBB-choline transporter. Since the BBB-choline transporter has not yet been crystallized, structural information about the protein binding site is not available. Therefore, establishing the relationship of the mapped CoMFA fields to the topography of the protein site with respect to the amino acid residues lining the binding pocket, is not possible. However, the synthesis of additional novel (i.e., rigid and/or bulky) ligands will continue to provide further insight into size limitations for ligands at the binding site about which the current model is unable to give clear insight. In addition, utilization of other molecular modeling approaches, such as pharmacophore analysis, may provide additional information. It is also important to point out that, as discussed above, the mono-azaaromatic quaternary ammonium compound, NONI, enters the brain via the BBB-choline transporter.[5,15] Furthermore, supporting evidence demonstrates that the bis-azaaromatic compound, bPiDDB, decreases self-administration of nicotine in rats after peripheral administration,[24] and is a potent inhibitor of [^{3}H]-choline transport via the BBB-choline transporter. Thus, taken together, these results suggest that both NONI and bPiDDB enter the brain via the BBB-choline transporter, and that CNS active compounds can be designed to utilize this transporter system as a portal of entry into the brain.

5. Conclusion

We have investigated a series of nAChR antagonists in earlier CoMFA and CoMSIA models of the BBB-choline transporter. Our earlier models could not adequately predict the BBB-choline transporter affinity of the novel bis-azaaromatic quaternary ammonium compounds. This prediction failure was likely due to the models not being able to accommodate the *n*-alkyl linkage separating the two cationic quaternary ammonium moieties, as these data sets did not include the molecular space encountered in these 'bis' compounds. A new model was constructed by including the bis-azaaromatic quaternary ammonium compounds into our previous data sets. The molecular models showed an excellent predictability value, which cross-validated at $q^2 > 0.4$. The current model was able to predict the affinity of hemicholinium-15, a 'monomeric' compound, which binds with much less affinity than hemicholinium-3, a structurally related 'dimeric' or 'bis' analog. From these studies it is evident that there is a need for the synthesis and evaluation of more rigid or conformationally restrained bis-quaternary ammonium analogs, to further determine the topographical architecture of the BBB-choline transporter binding site, and to elucidate the structural requirements for binding and transport.

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