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Research Paper

Non-Competitive Inhibitory Activities of Morphinan and Morphine Derivatives at the α3β4 Neuronal Nicotinic Acetylcholine Receptor Determined Using Nonlinear Chromatography and Chemometric Techniques

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Purpose. A series of morphine and morphinan derivatives were chromatographed on a column containing immobilized cellular membranes from a cell line expressing the α3β4 neuronal nicotinic acetylcholine receptor (α3β4 nAChR).
Methods. The results were analyzed using chemometric and molecular modeling techniques in order to predict the noncompetitive inhibitory (NCI) activity of these compounds, the molecular basis for the predicted activity and the binding sites of the inhibitors.
Results. The data demonstrated that seven of seven morphinans were NCIs and bound in the central lumen of the nAChR while only 2 of 13 morphine derivatives had NCI activity and these compounds most likely bound at the quinacrine binding site on the nAChR. The predicted activities were confirmed using functional inhibition studies.
Conclusions. The results indicate that this approach can be used to rapidly assess pharmacological activity and to guide new drug design.

KEY WORDS: affinity chromatography; functional assays; luminal binding site; molecular modeling; quinacrine binding site.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of the ligand gated ion channel family and are very abundant at the synaptic junctions of brain neurons (1). A broad range of endogenous and exogenous molecules interact with nAChRs modulating their synaptic activity. These agents may act directly on the neurotransmitter binding sites or allosterically on several other binding domains of the nAChR (2,3). The agonist binding sites are well characterized for most of the subtypes and are extensively utilized as molecular targets in medicinal chemistry and drug design (4,5).

The nAChRs also contain additional sites at which allosteric non-competitive inhibitors (NCIs) bind. These sites are located predominantly within the membrane portion of the receptor. The best characterized NCI binding site is located inside of the inner surface of the ion channel, the so called luminal domain, at which compounds bind and block the ion flux (3). A second site is located at the interface between the protein and membrane phospholipids and compounds such as *n*-alcohols, steroids or anesthetics bind at this site (6). Additional non-competitive binding sites have been identified for quinacrine (7) and ethidium (8).

The non-competitive inhibitory properties of a number of clinically useful drugs have been attributed to binding within the central lumen of the nAChR. These drugs include mecamylamine and dextromethorphan (9), bupropion and phencyclidine (10), ketamine (11) and barbiturates (12). Pharmacologically, the non-competitive inhibition associated with these drugs may be responsible for many of the clinically observed side effects. For example, the impairment of cardiovascular function during ketamine anesthesia has been associated with non-competitive inhibition of ganglionic nAChRs (11).

NCIs also present an opportunity for new drug development. The antidepressants sertraline, paroxetine, nefazodone and venlafaxine have been identified as potent NCIs and it has been suggested that nAChR subtypes in the brain could be targets for the development of new antidepressant drugs (13). The NCIs mecamylamine and bupropion are currently used in anti-smoking therapy (14) and the use of the NCI 18-methoxycoronaridine in combination with mecamylamine or dextromethorphan has been suggested as an approach to the treatment of opioid and stimulant addiction (15).

NCI activity has been routinely determined by measuring concentration-dependent effects on whole-cell currents or

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nicotine-induced $^{86}Rb^{+}$ efflux, yielding IC_{50} values (9–12). These approaches are time-consuming and exacting. We have recently reported an alternative method for the identification and characterization of NCI of the nAChR using nonlinear chromatography and chemometric techniques, (16–20). This approach has been applied to the screening of some parent drugs and their metabolites (19,20) but has not been used to screen larger cohorts of structurally related compounds.

The objective of this study was to use this technique to screen a group of structurally related compounds, the 20 morphine and morphinan derivatives presented in Fig. 1, in order to determine if NCIs could be identified within and between the groups. The affinity chromatography studies were conducted using a column containing immobilized cellular membrane fragments obtained from a cell line expressing the α3β4 nAChR. The chromatographic data was compared to the results from nicotine-induced $^{86}Rb^{+}$ efflux studies conducted using the same cell line used to create the affinity column.

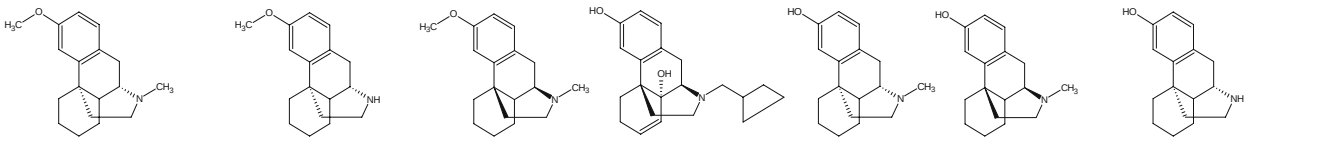
Morphinans and morphine derivatives were selected based upon previous data that demonstrated that the morphinans dextromethorphan ((+)-3-methoxy-*N*-methyl-morphinan), levomethorphan ((−)-3-methoxy-*N*-methyl-morphinan) and dextrorphan, the *O*-demethylated metabolite of dextromethorphan were potent NCIs of the α3β4 nAChR (19,21). This suggests that other morphinan derivatives as well as morphine derivatives may also be effective NCIs of the α3β4 nAChR. However, to our knowledge, the non-competitive inhibitory properties of morphine derivatives have not been established. The identification of non-competitive inhibitory properties of these agents would not only provide an insight into their efficacy and toxicity, but may also suggest pathways to new and more effective drugs.

MATERIALS AND METHODS

Materials

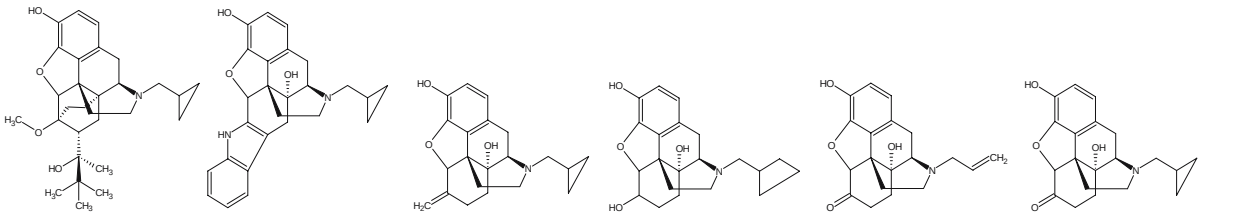
Buprenorphine hydrochloride, butorphanol tartrate salt, codeine, dextromethorphan hydrobromide, hydrocodone (+)-bitartrate salt, hydromorphone hydrochloride, levorphanol tartrate salt, morphine sulfate salt pentahydrate, nalbufine hydrochloride dihydrate, nalmefene, nalorphine hydrochloride, naloxone hydrochloride dehydrate, naltrexone hydrochloride, naltrindole hydrochloride, norcodeine, oxycodone hydrochloride, (−)-nicotine hydrogen tartate, and poly-D-lysine were purchased from Sigma (St. Louis, MO, USA). The dextromethorphan metabolites, dextrorphan, (+)-3-methoxy-morphinan and (+)-3-hydroxy-morphinan were kindly provided by Hoffman LaRoche (Nutley, NJ, USA). Levomethorphan was purchased from Cerilliant (Round Rock, TX). High-performance liquid chromatography grade methanol, ammonium acetate, 0.1 M ammonium hydroxide solution and other chemicals were purchased from Fisher

MORPHINANS

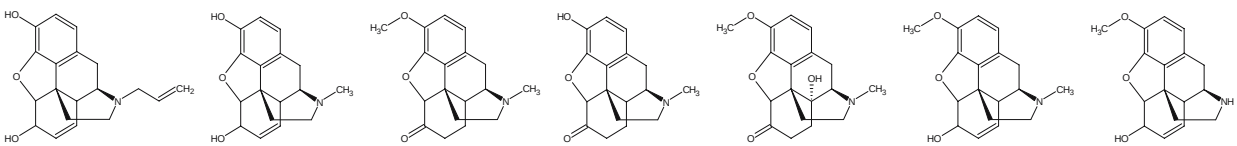


dextromethorphan (**1**) (+)-3-methoxy-morphinan (**2**) levomethorphan (**3**) butorphanol (**4**) dextrorphan (**5**) levorphanol (**6**) (+)-3-hydroxy-morphinan (**7**)

MORPHINES



buprenorphine (**8**) naltrindole (**9**) nalmefene (**10**) nalbufine (**11**) naloxone (**12**) naltrexone (**13**)



nalorphine (**14**) morphine (**15**) hydrocodone (**16**) hydromorphone (**17**) oxycodone (**18**) codeine (**19**) norcodeine (**20**)

Fig. 1. Structures of the compounds used in this study.

Table I. Functional and Chromatographic Data Obtained with the Compounds Used in this Study

	IC_{50} (μM)	k'	Log P
Morphinans			
1	10.1 (±1.1)[17]	96.2	3.67
2	10.4 (±1.1)	102.8	3.47
3	10.9 (±1.1)[17]	87.7	3.67
4	19.4 (±1.5)	33.3	3.82
5	29.6 (±5.7)[9]	41.6	3.42
6	39.9 (±1.0)	41.0	3.42
7	59.7 (±1.1)	40.8	3.22
Morphines			
8	12.6 (±1.3)	~270	3.49
9	27.5 (±1.1)	~300	3.82
10	~200	11.6	2.79
11	~200	7.8	2.09
12	~200	7.7	1.29
13	~250	6.5	1.49
14	~300	9.4	2.00
15	~300	6.3	1.39
16	~400	13.3	1.80
17	~400	9.0	1.55
18	~1000	5.9	0.92
19	~1000	7.1	1.64
20	>1000	6.1	1.43

The IC_{50} values were obtained using nicotine-stimulated $^{86}Rb^+$ efflux in the KXα3β4R2 cell line that expresses the α3β4 nAChR. The chromatographic retention factors, k's, were calculated using the equation $k' = t - t_0/t_0$, where: t = the observed retention expressed as minute and t_0 = the retention of a non-retained solute (water). The retention times were determined using immobilized membrane affinity liquid chromatography columns obtained from cell lines that expressed the α3β4 nAChR. See text for experimental details.

Scientific (Pittsburgh, PA, USA). The α3β4 nAChR column was prepared as previously described (18).

Tissue culture medium and penicillin/streptomycin were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Geneticin (G-418) was purchased from Cellgro by Mediatech (Herndon, VA, USA). Fetal bovine serum was obtained from Biosource International (Camarillo, CA, USA). ^{86}Rubidium chloride [$^{86}Rb^+$] was purchased from Perkin-Elmer (Boston, MA, USA).

Chromatographic Experiments

The chromatographic studies were carried out using Agilent LC/MS system series 1100 (Agilent, Palo Alto, CA) equipped with a vacuum degasser (G 1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20-μl injection loop, a mass selective detector (G1946 B) supplied with atmospheric pressure ionization electrospray, and an on-line nitrogen generation system (Whatman, Haverhill, MA). The apparatus was interfaced to a 250-MHz Kayak XA computer (Hewlett-Packard, Palo Alto, CA) running ChemStation software (Rev B.10.00, Hewlett-Packard). The mobile phase was composed of ammonium acetate (10 mM, pH 7.4)/methanol in a ratio of 85:15 (*v*/*v*), the flow rate was 0.2 ml/min, and the experiments were carried out at controlled temperature (20 ± 1°C). The ligands were monitored using their respective parent ions, $[M+H]^+$.

Nicotine-Stimulated $^{86}Rb^+$ Efflux Experiments on KXα3β4R2 Cells

The KXα3β4R2 cells were established and maintained as described previously (9). The function of nAChRs expressed in KXα3β4R2 cells was measured using the $^{86}Rb^+$ efflux assay (9). Briefly, cells in growth medium were plated onto 24- well plates. On the day of experiment, cells were loaded in medium containing 2 μCi/ml $^{86}Rb^+$ for 4 h at 37°C. After loading, cells were washed three times, and 1 ml of buffer with or without drugs was added to each well for 2 min. The efflux buffer was collected, and the cells were lysed in 1 ml of 0.1 N NaOH. The radioactivity in the efflux samples and cell lysates was measured by liquid scintillation counting. The amount of $^{86}Rb^+$ efflux was expressed as the percentage of the total $^{86}Rb^+$ loaded (fractional release). Stimulated $^{86}Rb^+$ efflux was defined as the difference between efflux in the presence and absence of nicotine (total efflux—basal efflux). For IC_{50} determinations, inhibition curves were constructed in which a range (0.03–500 μM) of concentrations for each antagonist was included in the assay to inhibit efflux stimulated by 100 μM

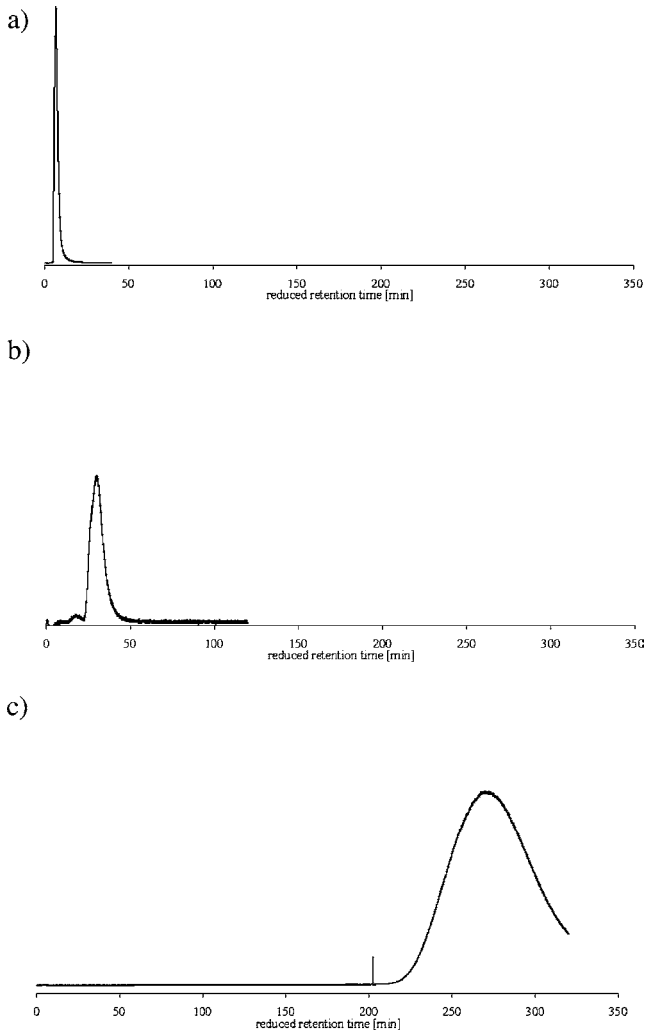


Fig. 2. Chromatograms obtained for three different opioids (a) codeine, (b) butorphanol and (c) buprenorphine on α3β4 nAChR affinity column.

nicotine. The IC_{50} values and curve fittings were determined by nonlinear regression analyses using Prism software (GraphPad Software, San Diego, CA).

MOLECULAR MODELING

Docking simulations were performed as previously reported (21). In brief, the molecular model of the luminal domain of α3β4 subtype nAChR was generated using a homology approach (21). The bundle of five transmembrane helices mimicking the inner channel of the nAChR resolved by the frozen state NMR method (21) was used as a homology template. AutoDock 3.0.5. was employed to develop the series of low energy orientations of conformationally free ligand molecule within the rigid structure of the protein binding site (21). The docking space was set to 22.5 × 22.5 × 45 Å to cover the whole binding area of the lumen. The modified genetic algorithm (GA-LS) in tandem with scoring function implemented in AutoDock (21) were employed to screen the docking space. Fifty docking runs were generated for each studied ligand and the lowest energy conformations were further used to compare the energies of docking and differences in complex orientations.

Molecular Surfaces Generation

Titan 1.0, (Wavefunction, Schrodinger) was used to generate the molecular surfaces of buprenorphine, naltrindole, quinacrine and ethidium. The initial structures of molecules were geometrically optimized with AM1 semi-empirical method and the electrostatic potential map over the density isosurface was generated. Ethidium molecule was modeled as a cation (total charge: +1).

RESULTS

Chromatographic Studies

The chromatographic retention of the morphinans, Fig. 1, compounds 1–7, used in this study ranged from 33.3 min (compound 4) to 102.8 min (compound 2) when they were chromatographed on the column containing the immobilized α3β4 nAChR, Table I. The chromatographic traces produced by these compounds were asymmetrical with significant tailing, c.f. Fig. 2a. These results are consistent with previous data for compounds 1, 2, 3, 5 and 7, obtained using a different immobilized α3β4 nAChR column(21).

The morphine derivatives used in this study are presented in Fig. 1, compounds 8–20. The chromatograms of the compounds 10–20 contained symmetric peaks, c.f. Fig. 2b, with retention times of less than 15 min, Table I. These results are consistent with previously reported chromatographic traces produced by compounds that had no affinity for the immobilized α3β4 nAChR (21). The chromatographic traces for the remaining two morphine derivatives, compounds 8 and 9, contained broad, asymmetric peaks with retention times of ~270 and ~300 min, respectively, Table I, Fig. 2c.

Functional Inhibition Studies

Of the seven morphinans used in this study, the IC_{50} values for compounds 1, 3 and 5 had been previously established

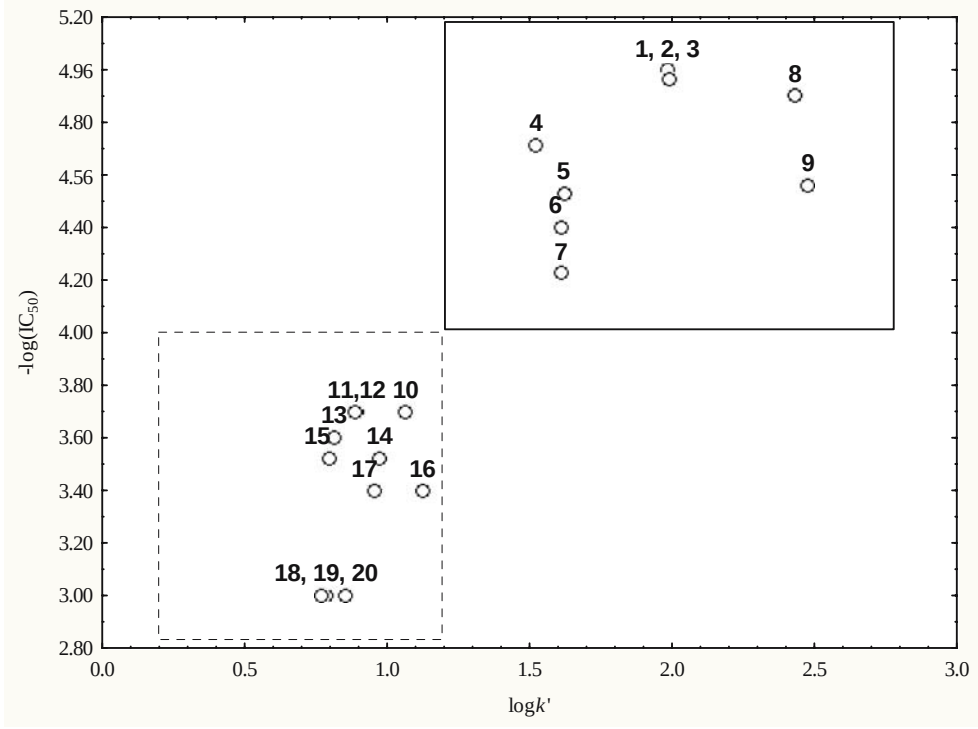


Fig. 3. The scatter plot of the −log (IC_{50}) values *versus* the log k' values for opioid ligands tested in α3β4 nAChR system. The whole cohort of compounds can be divided into 'non-NCIs' (*dashed line rectangle* IC50 >100 μM, and log k'<1.2) and 'NCIs' (*solid line rectangle* IC50 <100 μM, and log k' >1.2).

using the same experimental approach and cell line (9,19) and were not repeated in the study. Compounds 2, 4, 6 and 7 produced sigmoidal response curves and the calculated IC_{50} values ranged from ~10 μM (compounds 1, 2, 3) to 60 μM (compound 7), Table I.

Of the 13 morphine derivatives tested in this study, only compounds 8 and 9 produced sigmoidal response curves and the calculated IC_{50} values were 12.6 and 27.5 μM, respectively, Table I. All of the remaining compounds had no significant inhibitory effect on the nicotine-stimulated $^{86}Rb^+$ efflux in the test system and the calculated IC_{50} values were >200 μM, Table I.

Comparison of Data from Functional and Chromatographic Studies

The IC_{50} values obtained using nicotine-stimulated $^{86}Rb^+$ efflux in the KXα3β4R2 cell line were compared to the chromatographic retentions (k's) obtained on the column containing KXα3β4R2 cellular membranes, the α3β4 nAChR column. The two variables were compared by linear regression and the results were consistent with previous observations, which have demonstrated that k' is not a direct quantitative measure of functional properties, but rather a qualitative probe of IC_{50} (19) and EC_{50} (22).

Visual observation of the data contained in Table I suggested that the compounds used in this study fall into two distinct categories: 'high' IC_{50} (>100 μM) and 'low' IC_{50} (<100 μM). The threshold k' value for this classification appeared to be 15 (log k'=1.2), e.g., for all of the compounds with k'<15, the IC_{50} >100 μM and, conversely, for all of the compounds with IC_{50} <100 μM the observed k' was k'>15. A plot of log k' *versus* −log IC_{50} confirmed this observation, Fig. 3. A bimodal distribution of log k' was also observed with a threshold borderline between the two subsets of log k' equal to 1.2, i.e., k'=15 (data not shown). To validate this hypothesis a two-tailed Fisher's exact test was performed and a significant relationship, p=0.000, was observed between log k' and the grouping of IC_{50} values into 'high' or 'low' categories.

Chromatographic retention on the column containing KXα3β4R2 cellular membranes has been previously used to assess the IC_{50} values of 29 compounds relative to their ability to inhibit nicotine-stimulated $^{86}Rb^+$ efflux in KXα3β4R2 cells (19). In the previous study, the cohort of structurally varied NCIs was subjected to a three-dimensional cluster analysis based on log k' and the structural descriptors E_{HOMO} (the energy of the highest occupied molecular orbital) and S_{yz} (the surface area of the molecular projection onto the YZ plane). The method identified two clusters of compounds with IC_{50} values <7 μM (clusters 1 and 3) and one cluster of ligands with IC_{50} ≥10 μM (cluster 2). Using this analysis, compounds 1–7 and 10–20 were correctly placed in cluster 2, Table I, but could not be further segregated. The analysis presented in this paper is an extension of the initial clustering approach that can be used with families of closely related compounds. This approach did not place compounds 8 and 9 in cluster 2. They were placed instead in cluster 1 which contained NCIs such as verapamil and diltiazem.

DISCUSSION

The data from the functional studies demonstrate that all of the morphinan derivatives displayed some inhibitory effect on the nicotine-stimulated $^{86}Rb^+$ efflux, although it is reasonable to assume that this would be a pharmacologically significant property only for compounds 1, 2, and 3, Table I. This difference was also reflected in the chromatographic retentions of these compounds as compounds 1, 2 and 3 took more than twice as long to migrate through the column as did compounds 4–7.

In a reversed-phase chromatographic system, i.e. where the mobile phase is polar (aqueous) and the stationary phase is non-polar, non-specific hydrophobic interactions between a test compound (solute) and the stationary phase play a key role in the chromatographic retention. In this study, the stationary phase contained both hydrophobic interaction sites associated with the components of the immobilized membrane fragments and the stationary phase as well as polar interaction sites, including the nAChR which contains negatively charged glutamic acid moieties at the entrance to the central lumen. Thus, chromatographic retentions observed in this study were the summation of a variety of different specific and non-specific interactions occurring between the solute and the components of the stationary phase. In order to clarify the relationship between the chromatographic and functional data, it was important to determine the contributions of nonspecific hydro-

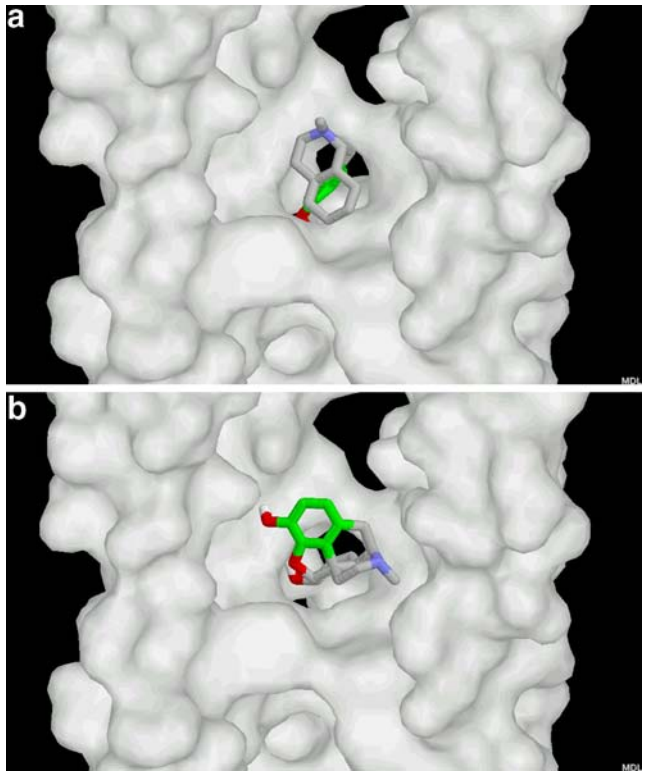


Fig. 4. Comparison of the orientations of complexes with the NCI active site of the α3β4 subtype nAChR for (a) levomethorphan and (b) morphine, simulated with AutoDock. The active site is rendered as a solvent-accessible surface; ligands are rendered in color-coded tube mode (nitrogen, *blue*; oxygen, *red*; aromatic carbon atom, *green*; and other carbon atoms, *grey*). For details of the docking procedure see reference.

phobic interactions and specific binding interaction with the nAChR to the observed differences in retention both within and between families.

For the morphinan derivatives, the data indicate that, while lipophilicity contributed to the observed retentions, specific interactions with the nAChR played a key role in this property. This is indicated by the fact that while compound 4 had the highest log *P* value, 3.82, of the morphinans, it had the lowest retention, 33.3 min, Table I. In addition, compounds 1 and 3 are enantiomers and therefore have the same physicochemical properties, including their log *P* value of 3.47. However, their retention times differ by almost 9 min, which has been previously shown to be related to differences in the disassociation rate constants of the diastereomeric NCI–nAChR complexes and not to the observed IC_{50} values (17).

Unlike the morphinan derivatives, non-specific interactions appeared to play a greater role in chromatographic retention of the morphine derivatives as a trend was observed between the log *P* values of compounds 10–20 and their respective k' values, $r^2 = 0.4428$ ($p = 0.025$), data in Table I. In addition, compounds 10–20 had no significant inhibitory effect on nicotine-stimulated $^{86}Rb^+$ efflux, Table I, suggesting that they do not interact with the α3β4 nAChR. Thus it is reasonable to conclude that compounds 10–20 had no significant interactions with the immobilized α3β4 nAChR which was also suggested by the symmetrical chromatographic peaks and retention times of <15 min.

While the significant differences in log *P* values ($p < 0.001$) between the morphinan compounds (log $P = 3.53 \pm 0.08$) and the morphine derivatives 10–20 (log $P = 1.67 \pm 0.15$) certainly play a role in the observed divergence in their inhibitory functions and chromatographic retentions, they do not appear to be the primary source of these differences. Rather, previous non-linear chromatographic studies of NCIs on the α3β4 nAChR column have identified the dissociation rate constant, k_d, of the NCI–nAChR complex as a key determinant of inhibitory function and chromatographic retention (16,20). In addition, Van't Hoff and molecular modeling studies using the enantiomeric compounds 1 and 3 demonstrated that the k_ds reflected the thermodynamic stabilities, ΔG^o, of the NCI–nAChR complexes (17,21).

The differences in k_d and ΔG^o appear to be based on the ligand–receptor binding mechanism that occurs within the luminal domain. Molecular modeling studies of the interactions between NCIs and the luminal domain of the α3β4 nAChR revealed the existence of a hydrophobic cleft in the non-polar region near the "V/F ring" (21). Simulated docking experiments using luminal NCIs suggested that stable NCI–nAChR complexes were the result of the insertion of

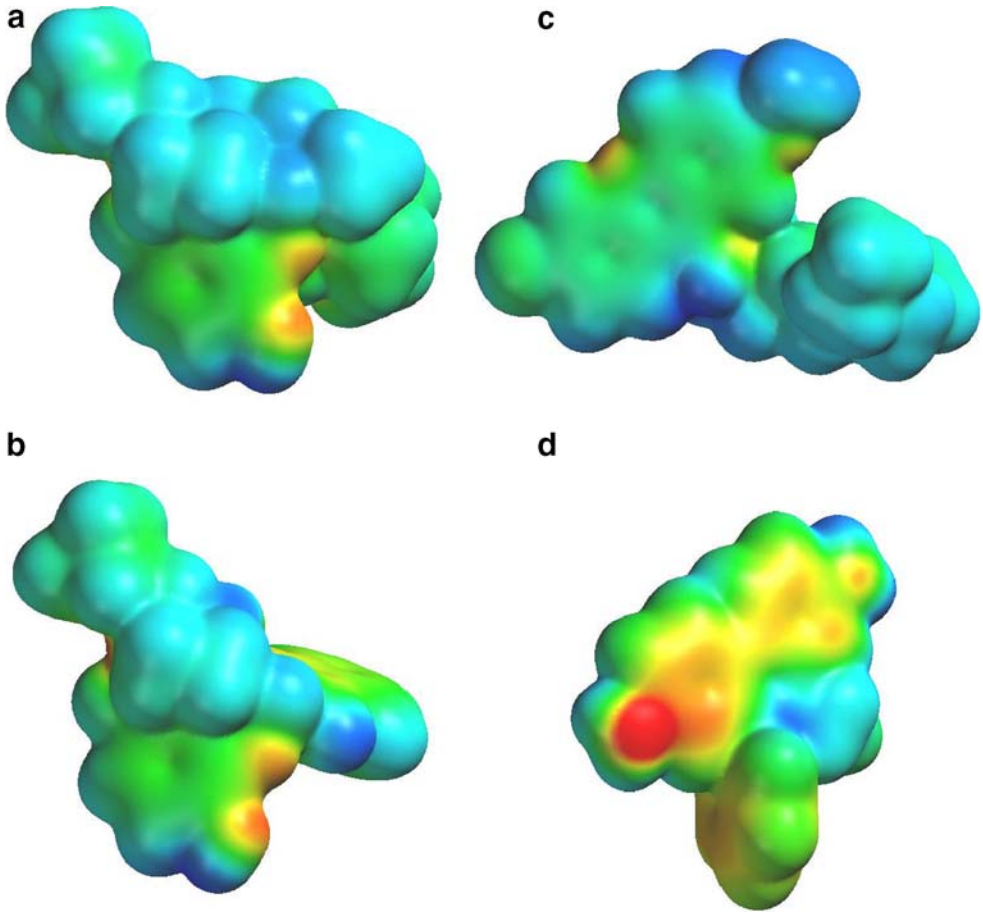


Fig. 5. Comparison of molecules representing compounds (a) 8, (b) 9 and (c) quinacrine and (d) ethidium. The electrostatic potential mapped on the molecular density isosurfaces for each molecule was generated with the Titan 1.0, as discussed in "Methods" section.

a hydrophobic portion of the inhibitor into the cleft accompanied by interactions between polar moieties on the NCI and polar residues exposed to the lumen, Fig. 4a. This binding mechanism may play a key role in the non-competitive inhibition of the α3β4 nAChR as binding at the lipophilic cleft increases the energy barrier between the closed and open states of the receptor, thereby hindering the conformational changes associated with nAChR function (18).

Docking experiments employing the morphinans (compounds 1–7) used in this study demonstrated that all of these compounds were able to bind at the hydrophobic cleft, c.f. Fig. 4a. When the morphine derivatives (compounds 8–20) were docked in the α3β4 nAChR model, these compounds did not significantly penetrate into the hydrophobic cleft, c.f. Fig 4b. Thus, the difference between the two families appears to be due to the epoxy bridge between positions 4,5 of the morphan ring system, which prevents the molecules from assuming the same orientation as the aromatic portion of the morphinan ring system relative to the hydrophobic cleft. Based upon these observations, it appears that the difference between the retention and non-competitive inhibitory activity of the morphinan and morphine systems arises from their relative abilities to form stable complexes with the receptor by binding at the hydrophobic cleft.

As with the other morphine derivatives, compounds 8 and 9 did not fit into the hydrophobic cleft. However, unlike compounds 10–20, compounds 8 and 9 had significant inhibitory activity, IC_{50} 12.6 and 27.5 μM, respectively. These compounds were retained on the α3β4 nAChR column for over 250 min and lipophilicity did not appear to play a significant role in the observed chromatographic retentions, c.f. log P values, Table I. Therefore, it appears that the conclusions drawn for compounds 1–7 and 10–20 are not applicable to these compounds.

In a previous study, when the NCIs ethidium and quinacrine were chromatographed on the immobilized α3β4 nAChR column, ethidium took over 190 min to elute from the column while quinacrine was determined to bind irreversibly to the column under the experimental conditions (21). These results were attributed to the fact that neither of these compounds bind at the V/F ring of the central lumen. The binding site for ethidium is postulated to reside in the outer vestibule of the central lumen about 46 Å above the transmembrane portion of the receptor (4) while the primary binding site for quinacrine has been identified in the "nonannular" lipid domain on the border between the nAChR and the membrane, and not in the luminal domain (4,7). The long retention times for compounds 8 and 9 coupled with their observed NCI activity suggest that these compounds preferably bind at either the ethidium or quinacrine sites.

Molecular models of the compounds used in this study were constructed and their molecular volumes and electrostatic potentials were compared. The molecular volumes of compounds 8 and 9 were 449 and 376 $Å^3$, respectively, as compared to 285 ± 8 $Å^3$ (compounds 1–7), 270 ± 10 $Å^3$ (compounds 10–20), 304 $Å^3$ (ethidium) and 388 $Å^3$ (quinacrine). Thus, the molecular volumes of compounds 8 and 9 are significantly closer to quinacrine than to ethidium or the other compounds used in this study.

The electrostatic potentials of compounds 8 and 9, quinacrine and ethidium were mapped on their respective isosurfaces, Fig. 5a–d, respectively, and the data demonstrate that compounds 8 and 9 possess the same predominantly hydrophobic surface present in quinacrine. These three compounds differ from ethidium, a polyamine, which displays a different electrostatic potential map.

The results suggest that compounds 8 and 9 resemble quinacrine and should be expected to bind within the nonannular lipid domain. This hypothesis was tested using displacement chromatography. It had been previously demonstrated that binding at the extracellular agonist binding site of an immobilized α3β4 nAChR could be distinguished from binding inside of the central lumen of the receptor through the determination of the effect on chromatographic retention on test compounds produced by the addition of either epibatidine (an agonist) or mecamylamine (a NCI) to the mobile phase (16). This experimental approach was employed in the current study through the addition of 10 μM quinacrine to the mobile phase. The introduction of quinacrine produced a 60% reduction in the retention of compound 8, a 98% reduction in the retention of compound 9 and a 28% reduction in compound 15 (negative control). These results are consistent with a competitive displacement of compounds 8 and 9 by quinacrine and support the supposition that these compounds bind primarily at the quinacrine binding site.

The assumption that compounds 8 and 9 primarily bind at the quinacrine binding site suggests that this may also be the principle binding site for other NCIs. The previously described cluster analysis apparently misplaced compounds 8 and 9 by putting them in the predominantly low IC_{50} (<7 μM) cluster 1. One of the parameters used in the cluster analysis is a molecular size parameter, Syz. The calculated Syz parameters for compounds 8 and 9 were 69.8 and 65.9 $Å^2$, respectively, which does not significantly differ from most of the other NCIs placed in cluster 1, for example the Syz for verapamil is 69.1 $Å^2$ and the Syz for diltiazem is 62.3 $Å^2$. Thus, like compounds 8 and 9, the NCIs contained within cluster 1 may primarily bind at the quinacrine binding site. This possibility is currently under investigation and the results will be presented elsewhere.

CONCLUSIONS

In this study, chromatographic and chemometric methods have been used to identify non-competitive inhibitors of the α3β4 nAChR in a series of morphinan and morphine derivatives and these predictions were confirmed using a functional assay of non-competitive inhibition. In addition, the results from molecular modeling studies indicated that the differences in inhibitory activity between morphinan and morphine derivatives arose from steric effects in the binding of the compounds to a hydrophobic pocket located within the central pore of the nAChR. The chromatographic and chemometric data also indicated that the non-competitive inhibitory properties of the morphine derivatives compounds 8 and 9 were the result of binding at the quinacrine binding site and not within the central lumen of the nAChR. This assumption was supported by the results of displacement chromatography experiments. The affinity chromatography approach in tandem with chemometric and modeling analysis

led to many important conclusions, which could not be drawn from regular pharmacological analysis of the dose response data alone.

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