using ligand-based approaches. A large number of ligand-based studies on NNRs have been conducted to explore NNR quantitative structure-activity relationships, identify pharmacophoric elements, and design novel and subtype selective NNR agents. Our studies will highlight examples of ligand-based modeling strategies of NNR ligands using a variety of methodologies (random forest, k nearest neighbors, Bayesian, shape and similarity based pharmacophore, etc.) with data based on orthosteric agonists and antagonists at α4β2, α7, α3β4 and/or α6β2* NNR subtypes. We will also briefly discuss application to ligand development for therapy.

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1.14

The twin drug approach for novel nicotinic acetylcholine receptor (nAChR) ligands: Synthesis and structure–affinity relationships

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We have used known nicotinic acetylcholine receptor (nAChR) ligands, as well as important elements of their pharmacophores, to design and synthesize novel nAChR ligands using the twin drug approach. Either two identical or two non-identical pharmacological entities were combined in different ways (linker, no linker, overlap). For example, we generated heterodimer ligands with one part derived from antioxidants, NSAIDs, scaffolds for monoamine related targets, or for beta-amyloid interaction and the second part derived from a nicotinic ligand. In a first approach to evaluate the effect of these compounds on diverse nAChRs, the compounds synthesized were tested for their affinities for different nAChR subtypes using the radioligands [^{3}H]epibatidine (α4β2*, α3β4* and muscle type nAChRs) and [^{3}H]methyllycaconitine (α7* nAChRs). We also tested these compounds on membrane fractions from rat brain, pig adrenals, and Torpedo californica electroplax in competition assays. A broad spectrum of affinities (e.g. Ki values for α4β2*: <10 nM to >10,000 nM) provided important insights into structure–affinity relationships. These studies will result in novel compounds that could ultimately be useful for development of therapeutics to treat disorders involving nAChR dysfunction.

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1.15

Scanning mutagenesis of α-conotoxin AuIB reveals a critical residue for activity at the α3β4 nicotinic acetylcholine receptor

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α-Conotoxin AuIB, a disulfide-bonded peptide of 15 amino acids with a 4/6 intercysteine spacing, inhibits the α3β4 nicotinic acetylcholine receptor (nAChR) subtype, which is a predominant subtype in the peripheral nervous system [1,2]. The ribbon isomer of AuIB has been shown to be more potent than the native AuIB (globular isomer) and to discriminate between stoichiometries of α3β4 nAChRs expressed in *Xenopus* oocytes [3]. AuIB also inhibits high voltage-activated N-type calcium channels in rat DRG neurons via the activation of G protein-coupled $GABA_B$ receptors [4]. Interestingly, α-conotoxin AuIB possesses analgesic activity *in vivo* and, therefore, may be a potential drug lead for treating chronic and neuropathic pain. In order to develop improved drugs void of side effects, it is necessary to understand the molecular determinants of AuIB binding to its putative targets: $GABA_B$ receptor vs. α3β4 nAChR. The aim of the present study was to determine the critical amino acid residues of AuIB responsible for its interaction with α3β4 nAChRs. Alanine scanning mutagenesis of the native AuIB peptide was carried out to construct AuIB alanine-substituted analogues which were tested in *Xenopus* oocytes expressing rat α3 and β4 subunits. Two-electrode voltage clamp recording was used to assess the effect of AuIB and its analogues (3 μM) on the ACh-evoked current amplitude. Phenylalanine to alanine mutation at position 9 of AuIB abolished inhibition of α3β4 nAChRs, whereas substitution of glycine at position 1 with alanine significantly reduced inhibition ($18.0 \pm 10.5\%$, $n = 3$) compared to native AuIB ($48.5 \pm 6.9\%$, $n = 7$) ($p < 0.05$). Mutation of residues other than cysteine and proline, which are known to disrupt the tertiary structure of α-conotoxins, did not significantly reduce the inhibition of ACh-evoked currents compared to native AuIB. Subsequent homology modelling/docking simulation was performed using a homology model of the rat $(\alpha3)_2(\beta4)_3$ nAChR. The results suggest that interaction of AuIB Phe9 with Lys81 and Trp79 on the β4 nAChR subunit may be essential for AuIB binding/interaction on α3β4 nAChR. In conclusion, we have identified phenylalanine at position 9 as the critical residue for specific interaction of AuIB with the α3β4 nAChR. Future studies using site-directed mutagenesis of the β4 subunit are required to further dissect the mechanism of AuIB binding/interaction on α3β4 nAChR.

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1.16

Acetylcholine binding protein-nicotinic receptor chimeras for delineating structure and determinants of ligand selectivity

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Acetylcholine binding proteins have provided a wealth of information on structure of the extracellular domain of the Cys-loop ligand-gated ion channels since their initial report by Sixma and colleagues. The availability of high resolution X-ray crystal structures of these proteins in complex with various nicotinic ligands has provided an atomic resolution view of the determinants of ligand recognition. In turn, this has provided opportunities for

structure-guided drug design, target "templated" synthesis and computational analyses of ligand recognition. However, these efforts have been hampered by the fact that the binding proteins, while homologous with human nicotinic receptors, have low overall sequence identity and limited state changes, resulting in a pharmacology that is dissimilar to human drug targets. To address this shortcoming we have designed chimeric binding protein constructs in which the C loop and other segments of the binding site have been replaced with the amino acids corresponding to the Cys-loop receptors. In an initial step to developing surrogates of human extracellular domains amenable to crystallography, we modified the C loop and examined the ligand selectivity changes and X-ray crystal structures of these chimeras to assess the utility of the chimera approach in high throughput screening and *in situ* freeze-frame click chemistry. In addition to these constructs providing an interesting prospective on the role of the C loop in ligand recognition and specificity, they have in some cases provided X-ray crystal structures of ligands that hitherto have been difficult for us to obtain. Among these are some touchstone ligands currently on the market or under clinical investigation including varenicline (Chantix®), sazetidine A and cytisine and natural toxins, such as anatoxin A. Accordingly, the C loop and how it is configured in the ligand complex are determinants of crystal nucleation and growth. Details on the comparative structures of the above ligand complexes provide details on the determinants of ligand selectivity for receptor subtype and offer insights into the development of more selective agonists and antagonists.

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1.17

Pharmacological properties of sazetidine A, a selective ligand of α4β2 nicotinic acetylcholine receptors

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Neuronal nicotinic acetylcholine receptors (nAChRs) serve a wide range of physiological functions and are implicated in a number of pathological processes and many pharmacological effects of nicotinic drugs. In particular, several lines of evidence indicate that the nAChRs containing both α4 and β2 subunits mediate important *in vivo* effects of nicotine, including its addictive and cognitive effects. Sazetidine-A (Saz-A) selectively binds with high affinity to α4β2 nAChRs and shows potent *in vivo* effects in animal models that include analgesia, reduction in nicotine self-administration, reduction in alcohol intake, antidepressant-like activity and reversal of attentional impairment. In *in vitro* studies, Saz-A potently inhibits nicotine-stimulated ion efflux from cells that express α4β2 nAChRs after they are pre-incubated for 10 min with Saz-A [1]. Saz-A shows full agonist activity at $(\alpha4)_2(\beta2)_3$ nAChRs but little agonist activity (<1% efficacy of that of acetylcholine) at $(\alpha4)_3(\beta2)_2$ nAChRs expressed in *Xenopus* oocytes [2]. Hence, an important question is how each of these two essentially diametrically opposed actions of Saz-A, activation and desensitization, contributes to each of the *in vivo* effects of Saz-A. We hypothesize that Saz-A converts most of the receptors to a desensitized conformational state after a brief exposure to it. Using equilibrium and kinetic binding methods, ion efflux measurements and patch-clamp electrophysiology, we compared *in vitro* pharmacological properties of Saz-A with those of nicotine, epibatidine, varenicline, 5-I-A-85380 and cytisine. Based on data from these *in vitro* studies and observations obtained from studies in behavioral animal models, we hypothesize that the long-lasting, selective desensitization of α4β2 nAChRs is the main mechanism for long lasting *in vivo* effects of Saz-A.

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1.18

The $(\alpha4)_3(\beta2)_2$ nAChR has a benzodiazepine-like modulatory binding site in the αα-subunit interface as revealed by studies with NS9283

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Modulating α4β2 nicotinic acetylcholine receptors through novel allosteric binding sites represents an exciting new area for pharmacological intervention of higher brain function including attention and cognition. NS9283 was originally indentified by its ability to increase agonist-evoked response amplitude of α4β2 nAChRs in Ca^{2+}-imaging as well as in electrophysiology paradigms. NS9283 did not itself produce receptor activation or displacement of [^{3}H]-epibatidine binding and the allosteric modulation was found to be selective for α2- and α4-containing nAChRs whereas no effects were observed at α1-, α3- or α7-containing nAChRs. α4β2 nAChRs are known to assemble as high- or low-sensitivity receptors dependent on subunit stoichiometry and in *Xenopus* oocyte experiments NS9283 modulation only occurred when receptors were expressed under conditions favoring a $(\alpha4)_3(\beta2)_2$-subunit stoichiometry indicating that NS9283 is selective for low-sensitivity receptors. We therefore hypothesized that the selectivity was dependent on a 3α:2β-subunit stoichiometry and in particular on the αα-subunit interface. Comparing homology models we have identified amino acids that could be involved in binding of NS9283. Of these, Histidine 142, located on the (−)-side of the α4-subunit, was particularly interesting since NS9283 is devoid of any effects up to a concentration of 31.6 μM on $(\alpha4^{H142V})_3(\beta2)_2$ receptors. Studies investigating the mode of action of NS9283 revealed that modulation of i.e. $(\alpha4)_3(\beta2)_2$ receptors could be attributed to an increase in functional agonist potency but maximal current amplitude were unaffected. Graphically, this is seen as left-shift of agonist concentration-response curves, towards higher potency of the agonist, but maximal efficacy is not affected. The key features of NS9283, i.e. subunit interface binding and left shift of agonist concentration–response curves without affecting efficacy, resembles those described for benzodiazepines at the benzodiazepine binding pocket of $GABA_A$ receptors. We therefore propose that NS9283 is a mimic of a benzodiazepine mechanism in the nicotinic system. In conclusion, NS9283 demonstrates that it is possible to find highly selective allosteric modulators of nAChRs and further