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Contemporary paradigms for cholinergic ligand design guided by biological structure

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Abstract—The identification of the various nicotinic receptor subtypes, when coupled with the recent development of three-dimensional structures of surrogate extracellular receptor domains, offers new opportunities to design nicotinic ligands. Conformation and fluctuations in receptor structure are critical to ligand selectivity, and we present here how a flexible receptor template can be used in the development of selective ligands affecting cholinergic neurotransmission.

Close to a century ago Langley and his student Elliot proposed that cellular communication within the nervous system occurred through chemical neurotransmission. A necessary corollary of their proposal is the existence of receptors, termed by them as 'receptive substances'.[1] For the first 70 years, receptors were studied by inference through structure–activity relationships, enantiomeric selectivity, agonist-antagonist relationships and, somewhat later, covalent modifications to influence responsiveness. Only in the last 30 years, with the discovery of α-bungarotoxin to identify and characterize the nicotinic acetylcholine receptor,[2] have we witnessed the treatment of the receptor as a discrete chemical entity. A century following Langley's studies in this genome-based era a new paradigm has emerged, where a structural template or surrogate structure does or will soon exist for all receptors and biological targets.

Sequencing the genome yielded a potential primary structure for all of the targets of drug action, and technical advances in crystallography and NMR spectrometry have produced structures of the gene products or individual domains of gene products at atomic level resolution. In some cases the actual receptor target has been characterized structurally;[3] in others, a homologous surrogate target has a resolved structure.[4–6] Accordingly, these structures, similar to lead compounds in small molecule design, provide templates for drug discovery. However, sequence differences between members of homologous proteins, structural constraints imposed by the crystallization process and the recognition that the ligand itself may induce changes in structure are considerations that require continuous refinement of the template.

An appropriate example comes from considerations of the nicotinic acetylcholine receptor (nAChR), where a recently crystallized homopentameric acetylcholine binding protein (AChBP) isolated from the fresh water snail *Lymnaea*, was shown to be homologous to the N-terminal 210 amino acids in the nAChR.[5,7] As a soluble entity homologous to the extracellular domain of the receptor, but lacking in its transmembrane spans, the protein can be purified and crystallized. We have synthesized the encoding gene and expressed this protein from *Lymnaea* as well as from the saltwater species, *Aplysia*.[8,9] The sequences and some physical properties of the two proteins differ substantially. When agonist and competititive antagonists bind to the protein, substantial alterations in the native tryptophan fluorescence occur, and fluorescence can be used to monitor equilibrium binding, stoichiometry and kinetics of association directly.[8] The latter occurs, as it does for the receptor, in the millisecond time frame requiring stopped-flow instrumentation. Moreover, for a soluble protein, several physical techniques can be employed to correlate conformation with ligand association in AChBP. This protein most closely resembles the α7 nAChR,[7] and it should be possible through chimera formation and residue substitution to construct an extracellular domain of the α7 receptor to examine ligand specificity and

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conformation. Already, the expressed binding proteins themselves are yielding interesting ligand selectivity differences that should ultimately prove useful in analyzing recognition mechanisms and conformation of the binding site. For example, the *Aplysia* binding protein has 14,000-fold selectivity for α-conotoxin Im1, whereas α-bungarotoxin is selective for the *Lymnaea* binding protein.[8,9] The expression of the receptor as a soluble assembled pentamer in milligram quantities not only enables one to conduct detailed physical studies, but also to develop high throughput assays for ligand screening.

Advances in resolving the structures of potential drug targets have been paralleled by developments in computation of structural perturbations and fluctuations through molecular dynamics. However, a time range exists where a paucity of experimental data exists on the dynamics of proteins, and this extends from the range of molecular events associated with function, such as channel opening, on one extreme to the measurement of molecular dynamics on the other extreme. As shown in Figure 1, channel opening and closing processes are resolvable down to 100 μs; a similar time frame is found for the deconstructed catalytic steps of our most efficient enzymes such as acetylcholinesterase. Computation through molecular dynamics, if H_2O is treated as an explicit solvent and computational power is high, extends up to tens of nsec. The four order of magnitude gap between 10 nsec and 100 μs can be subserved by several techniques to provide time-resolved information on conformation.

With David Johnson at UC, Riverside, we have employed fluorescence lifetimes and time-resolved fluorescence anisotropy to that end for both AChBP and acetylcholinesterase.[10,11] By substitution of cysteines at designated positions in the molecule, it is possible to label selectively designated side chains and analyze both solvent exposure at equilibrium and dynamic fluctuations in molecular structure as a function of position of the label and occupation of the ligand.[10,12] From this approach, changes in the microscopic environment around the protein surface and dynamic structural fluctuations associated with ligand binding can be analyzed. Hence, part of the knowledge gap in the nsec time frame is satisfied with time-resolved fluorescence techniques. Nevertheless, this is a far cry from achieving a comprehensive picture of protein dynamics. In the case of acetylcholinesterase, it has been possible to correlate the fluctuations seen with the decay of anisotropy with the vectoral movements of the protein.[10] Moreover, the data indicate that crystal structure tends to lock the system into a single, closed gorge conformation, and the dynamics show the departure to the various extant conformational states in solution.

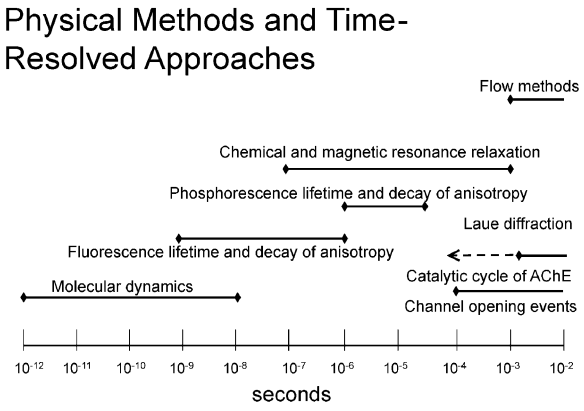

Figure 1. Time scale for accessible time-resolved structural data. Typically channel opening and closing events with receptors can be resolved down to 100 μs, and many physical and kinetic techniques allow for time resolution down to this range. At the other end of the scale, computational techniques using molecular dynamics and considering explicit H_2O molecules allow for calculations up to 10 nsec. The figure shows some of the techniques that cover the gap between these time scales.

The two premises that: (a) structural knowledge of all drug targets at atomic level resolution is potentially accessible, and (b) proteins are fluctuating dynamic molecules with multiple conformational states lead to further considerations in the design of selective molecules. The first is that conformations induced by ligand binding may depart substantially from the structure of the unliganded protein and may vary with the structure of the ligand. If that is the case, it becomes worthwhile to consider use of the macromolecular template itself to synthesize the ligand from bound precursors. The imaginative chemistry of Barry Sharpless and his colleagues leads to that end.[13] Acetylcholinesterase contains two discrete binding sites for inhibitors: one exists at the active center at the base of the gorge some 20 Å in depth; the other resides at the rim of the gorge. By extending aliphatic chains from the two binding molecules terminating in either an acetylene or azide moiety, a cycloaddition coupling reaction occurs leading to the formation of a *syn* or *anti* substituted 1,2,3-triazole. The reaction, using a combinatorial library of reactants and carried out on the surface of the molecule, appears specific for chain length and for regiospecificity yielding a single *syn*-regioisomer. Its characterization by DIOS mass spectrometry shows the synthesis of a single regioisomer in near stoichiometric quantitites with the enzyme.

The *syn*- and *anti*- configurations of the triazole appear in equivalent yields when synthesized in the absence of the enzyme template, and we find that the prevalent *syn*-compound, when formed on the AChE surface in situ, has, by far, the higher affinity for its binding site. Moreover, exceedingly high affinities in the femtomolar range are achieved rivaling the highest affinity natural and synthetic complexes.[14] Subsequent analysis of the crystal structures of the two complexes by Pascale Marchot and Yves Bourne in Marseille yields an additional surprise. Whereas the lower affinity, *anti*-complex yields a complex with little perturbation of structure from the apo-enzyme, the *syn*-complex shows major perturbations in the vicinity of the gorge constriction where the triazole lies and at the gorge rim where the propidium anchor resides.[15] Accordingly, high affinity complexes may exist in conformational states differing substantially from the apo- or unliganded protein.

Hence through 'click chemistry' selectively forming a cycloaddition product, the potential exists for develop-

ing freeze-frame inhibitors where either the reactants may induce unique conformations in the enzyme or the proximity of the reaction pair enables one to select unique conformations of the drug target. The proof of principle for acetylcholinesterase shows that one can achieve both high affinity and selectively for the enzyme species and great selectivity for ligand structure. More important therapeutic outcomes may emerge with the nicotinic receptor where selectivity of binding sites is desired among the vast array of receptor subtypes.

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