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COMMUNICATION

Covalent attachment of antagonists to the α7 nicotinic acetylcholine receptor: synthesis and reactivity of substituted maleimides†

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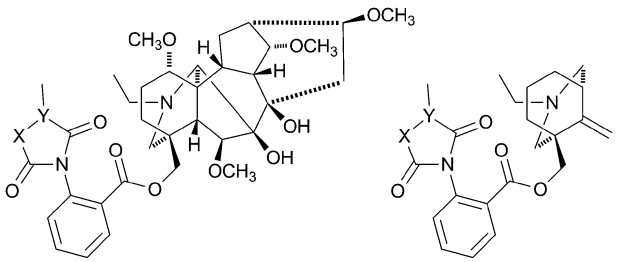
The 3-methylmaleimide congeners of the natural product methyllycaconitine (MLA) and an analogue covalently attach to functional cysteine mutants of the α7 nicotinic acetylcholine receptor (nAChR).

Bioconjugation provides a powerful tool to interrogate biological systems through the chemoselective and site-specific covalent attachment of ligands to biomolecules.[1–3] A powerful example of this is the Substituted Cysteine Accessibility Method (SCAM),[4–6] which employs cysteine mutagenesis in combination with thiophilic reagents to investigate and modify proteins. The technique is most commonly used with water soluble methanethiosulfonate reagents to identify the solvent accessible residues of a protein, but related cysteine mutagenesis techniques have also been used to append affinity tags and chromophores, or to identify ligand binding sites using a range of electrophilic functionality including maleimides, haloacetamides or mustards.[7] We recently employed a thiophilic mustard derivative to establish the channel pore binding site of a novel azabicyclic ligand at the α4β2 nAChR.[8]

To further explore the potential of cysteine mutagenesis as a technique to probe ligand binding we elected to explore the norditerpenoid alkaloid methyllycaconitine (MLA) **1** which is widely used as a potent and subtype selective antagonist of the α7 nAChR. Despite the rapidly emerging structural data from related full length receptors[9] and X-ray crystal structures of mollusc-derived acetylcholine binding proteins (AChBPs) as models of the nAChR N-terminal region,[10] limited direct evidence is available on the nature of ligand binding at functional nAChRs. The binding mode of MLA **1** at the α7 nAChRs has recently been suggested from X-ray crystal structures of wildtype and mutant AChBPs.[11] In this study we employ cysteine mutagenesis in combination with thiophilic ligands to explore the binding of MLA **1** and a simple analogue **2** at functional α7 nAChRs.

To develop thiophilic modifications of MLA **1** and analogue **2** the exchange of the side chain methylsuccinimide with a substituted maleimide to afford compounds like **3** and **4** suggested itself as a suitably conservative substitution. Maleimides react rapidly, selectively and irreversibly[12] with thiolate-bearing residues such as the amino acid cysteine, even in the presence of other nucleophiles such as lysine.[3] This reaction irreversibly forms a thioether linkage between the maleimide and the cysteine residue.[6] Unsubstituted maleimides are routinely employed, and bromo-substituted maleimides[13] have recently been investigated, however other substituents have not been explored. Consequently we undertook preliminary studies to synthesise some substituted maleimides and explore their reactivity with thiol containing amino acids.



X–Y, CH_2–CH = methyllycaconitine (MLA) **1**; analogue **2**
X–Y, CH=C = MLA maleimide **3**; analogue maleimide **4**

The reaction of two model thiol amino acids, *N*-Boc-Cys-OMe and *N*-Ac-Cys-OH, was investigated with a range of substituted maleimides appended to a model sidechain ester (**5–9**, Scheme 1) under pseudo first order conditions. The maleimides were selected to afford a range of steric and electronic influences on reactivity and included the unsubstituted maleimide **5** together with the bromo **6**, methyl **7**, trifluoromethyl **8** and dimethyl **9** maleimides. Two synthetic routes were devised to access these compounds. The synthesis of maleimides **7** and **9** was accomplished by the DCC coupling of the appropriately substituted maleimide **10** with adamant-1-ylmethanol to give the desired esters. An alternate route was employed to access maleimides **5**, **6** and **8**. A DCC

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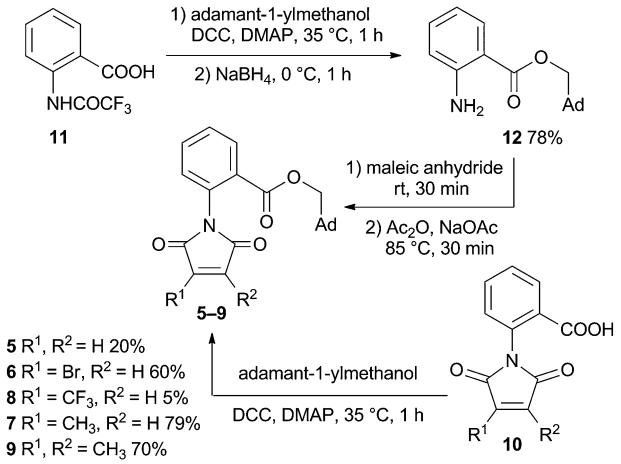
† Electronic supplementary information (ESI) available: experimental and computational procedures and data, PDB files for Fig. 2, ^{1}H and ^{13}C NMR spectra for compounds **3** and **4**. See DOI: 10.1039/c2cc32442c



Scheme 1 Synthesis of model maleimides.

coupling/deprotection sequence from acid **11** afforded anthranilate ester **12**. Subsequent condensation with the appropriate maleic anhydride gave the desired maleimides, albeit in a lower yield.

Reaction of each maleimide with a 10-fold excess of thiol-containing amino acids was conducted and maleimide consumption was monitored by integration of the ^{1}H NMR spectrum. Confirmation of conjugate formation was obtained by MS. Reaction with *N*-Boc-Cys-OMe as the thiol in CD_3OD established a relative rate of reaction as **5**/**6** > **7** ≫ **9** (no reaction). The reactions with **5** and **6** were judged complete prior to obtaining the NMR spectrum (3 min), while **9** showed no reaction after 24 h by ^{1}H NMR or MS. Reaction of *N*-Ac-Cys-OH as the thiol in d_6-DMSO established the relative rate of reaction as **5**/**8** > **6** ~ **7** ≫ **9** (no reaction). For **5** and **8** the reaction was judged complete prior to obtaining the first NMR spectrum (3 min), while **9** showed no reaction by NMR or MS after 24 h. The bromo **6** and methyl **7** maleimides required 6 h before complete consumption of the alkene proton was observed. The relative rates are comparable to other reports[13] and open avenues for the controlled deployment of substituted maleimides as Michael acceptors in bioconjugation chemistry.

Based on the reactivity profile, ease of synthesis and close structural resemblance to the methylsuccinimide present in MLA **1** and analogue **2**, the methylmaleimide was selected for the development of electrophilic probes. Methyllycaconitine maleimide **3** and analogue maleimide **4** were prepared by carbodiimide coupling in one step from lycoctonine[14] and azabicyclic alcohol[15] respectively in moderate yields.

From a homology model of the extracellular domain of the rat α7 nAChR built with MLA **1** in the binding site (see the ESI), two residues located on the complementary face of the subunit interface S188 and S189 were selected for cysteine mutagenesis. Further, to ensure that rapid desensitisation of the α7 nAChR did not result in erroneous measurements, a pore mutation changing the central leucine (L9′T) was introduced.[16] This mutant increased the sensitivity of the receptor to ACh (EC_{50} = 94 μM wt, 1.3 μM L9′T) but did not significantly alter the IC_{50} of MLA (IC_{50} = 238 pM wt, 228 pM L9′T). The S188C and S189C mutants were created from this L9′T background. When ACh was applied to oocytes expressing these receptors, a cation-selective current was elicited that was inhibited by MLA **1**, demonstrating that these receptors were functional.

Incubation of 1 μM MLA maleimide **3** with the L9′T receptor for 6 min did not alter currents elicited by ACh ($I_{(\infty)}$ = 1.09 ± 0.08, n = 4, Fig. 1). After 6 min incubation of 10 nM MLA maleimide **3**, the current elicited by ACh was significantly and irreversibly reduced in oocytes expressing S188C : L9′T compared to L9′T alone ($I_{(\infty)}$ = 0.24 ± 0.09, n = 3, p < 0.01). In contrast, the ACh response of S189C : L9′T was not significantly reduced by MLA maleimide **3** compared to the L9′T ($I_{(\infty)}$ = 0.85 ± 0.06, 1.12 ± 0.18 respectively, n = 4). The first order reaction rate constant for the reaction of MLA maleimide **3** to S188C : L9′T was estimated to be k = 0.0058 ± 0.0014 s^{-1}. This rate of binding and reaction is comparable to the estimated k_{on} = 0.0205 s^{-1} for binding of 10 nM [^{3}H]MLA to the α7 nAChR from rat membranes.[17]

Incubation of 1 μM analogue maleimide **4** with the L9′T receptor for 4 min reduced the currents elicited by ACh ($I_{(\infty)}$ = 0.88 ± 0.06, n = 3, p < 0.05 z-test *cf.* 1, Fig. 1). This reduction may result from slower wash out of **4** compared to MLA maleimide **3**, or alternatively, from reaction of **4** with the only other unpaired but sterically hindered cysteine in the extracellular domain, C138. After 4 min incubation of 200 nM analogue maleimide **4**, the current elicited by ACh was significantly and irreversibly reduced in oocytes expressing S189C : L9′T compared to L9′T alone ($I_{(\infty)}$ = 0.60 ± 0.04, n = 3, p < 0.05). In contrast, the ACh responses of S188C : L9′T were not significantly reduced by analogue maleimide **4** compared to L9′T alone ($I_{(\infty)}$ = 0.86 ± 0.09, n = 3). The first order rate constant for the reaction of analogue maleimide **4** to S189C : L9′T was estimated to be k = 0.011 ± 0.001 s^{-1}. These results suggest that MLA **1** and the analogue **2** bind with similar but not identical binding modes or alternatively, that Loop-F on which S188 and S189 lie may have different conformational preferences depending on which ligand is bound. In the rat α7 nAChR homology model (Fig. 2, cyan), S189 is hydrogen bonding to the succinimide moiety, with Cα 3.8 Å from the unsubstituted carbon of the succinimide moiety of MLA, and thus is positioned to react with maleimide analogue **4** after introduction of a cysteine mutation. In

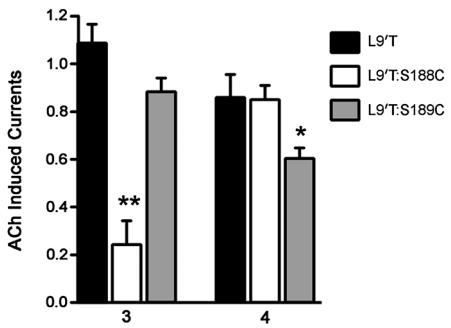


Fig. 1 Inhibition of ACh-elicited current by MLA maleimide **3** and analogue maleimide **4** at L9′T (1 μM each, 1 μM ACh, black bars) and mutant nAChRs (10 nM **3** and 200 nM **4** respectively, 5 μM ACh); S188C : L9′T, (grey bars) and S189C : L9′T (white bars). Inhibition is shown as the mean ± sem relative current elicited by ACh after incubation with the compound is complete.

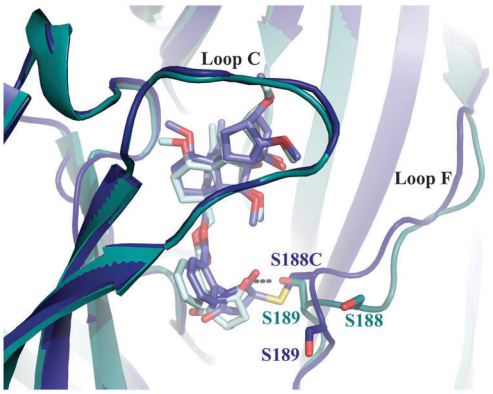


Fig. 2 Homology model of MLA **1** (pale cyan) bound rat α7 nAChR (cyan) with S189 to succinimide hydrogen bond indicated (dotted line), and loop sampled model (blue) after *in-silico* S188C mutation and reaction with MLA maleimide **3** (light blue).

contrast, the S188 Cα is placed 7 Å from the succinimide moiety and the side chain points away from the binding site. Rather than assuming a binding mode for MLA maleimide **3** different than that of MLA **1** in the X-ray structures,[11] the reactivity of the S188C : L9′T mutant may suggest that Loop-F is flexible and can change conformation so that S188 comes in close proximity of the succinimide/maleimide moiety.

To illustrate this possibility without violating common geometric restraints for the protein backbone, Loop-F was sampled using the Prime[18] extended loop sampling protocol (see the ESI) with a 3 Å distance constraint between the oxygen of S188 and the C4 of the succinimide moiety. As a result, a loop sampled model with S188 stabilised by hydrogen bonding to the oxygen of the succinimide moiety was obtained with a Ramachandran plot of a quality comparable to that of the original model. The loop-sampled model is shown in Fig. 2 (blue) for the S188C mutant following addition to MLA maleimide **3** and energy minimisation. While the exact position and functional importance of Loop-F in nAChRs is uncertain, there is substantial experimental support for Loop-F flexibility, *e.g.* different loop conformations in AChBP structures deposited in the RCSB Protein Data Bank,[19] high B-values and low electron densities.[20] Further, the length of Loop-F is different in the α7 nAChR receptor compared to the templates. Thus the loop-sampled model suggests an alternate F-Loop conformation supported by reaction data that is distinct from that observed in the original model and X-ray crystal structures.[11]

In conclusion, we have synthesised and studied the reactivity of five alkene-substituted maleimides (**5–9**) as Michael acceptors. From this work, thiophilic reagents **3** and **4** were developed from the known α7 nAChR antagonists MLA **1** and the simplified analogue **2**, and these were shown to covalently attach to cysteine mutants selected based on a structural model. This study of functional nAChRs using cysteine mutagenesis in combination with thiophilic ligands complements and confirms information afforded from recently reported AChBP crystal structures. The study reveals differences in reactivity that suggest different binding modes or more likely, different Loop-F conformations depending on which ligand is bound. The reactive probes **3** and **4** provide further scope to explore the nature of ligand binding to the α7 nAChR and other receptor subtypes. Our results detailing the interaction of MLA **1** with the α4β2 nAChR will be reported in due course.

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