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9.2.1.3 A Tale of Two Sites: the Controversy of Allosteric Versus Lumenal Binding for M2 Inhibitors and Implications for Drug Development

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The clustering of amantadine resistance mutations within the M2 TMD,[5,7,103,137,138] combined with early biophysical studies[139] and the first structures for the TM domain,[125] led to the commonly held view that both amantadine and rimantadine bound within the lumen in close proximity to His37, thereby preventing proton transport by occlusion or *via* interfering with associated steric changes in the His37/Trp41 tetrads. However, the first solution structure for the conductance domain sparked considerable controversy and renewed interest surrounding the mechanism by which these classical antivirals bound to the M2 channel complex.[80] In place of a single adamantane molecule in the lumen, four were located on the channel periphery bound to a pocket formed by the C-terminal region of the TMD and the beginnings of the amphipathic helices. This binding site was positioned so as to be readily accessible to rimantadine molecules partitioned within membranes, a phenomenon for which these compounds are well known.[140] In this scenario, the hydrophobic adamantyl cage resides in the hydrophobic region of the bilayer alongside phospholipid side chains, with the polar amine partitioned with head groups at the membrane–aqueous interface. However, a study was published in the same issue of *Nature* describing a TM domain crystal structure at low pH where density corresponding to amantadine was located within the channel lumen.[78] These parallel reports have created an upsurge in M2-related publications attempting to resolve this controversy in addition to renewed interest in M2 as an antiviral target.

The mechanism underpinning inhibition of M2 *via* binding at the allosteric site was proposed to involve stabilisation of the closed state of the channel, with drug binding preventing the conformational changes in the TM helix necessary for opening. In support of this, several well-known resistance mutations were seen to destabilise the tetramer, making drug binding less likely.[127] In addition, magic angle spinning NMR studies on conductance domain peptides in bilayers supported that rimantadine bound the channel at a stoichiometry greater than one and interacted with residues corresponding to the allosteric site.[141]

However, a large number of studies have argued against binding of adamantanes to the M2 periphery. These include functional studies where chimeric proteins were generated between the M2 proteins of influenza A (AM2) and that of influenza B (BM2), which is not sensitive to amantadine or rimantadine.[142] Transfer of part of the AM2 N-terminus and TM domains (aa 19–36) into BM2 conferred amantadine susceptibility in oocyte membranes, arguing that drug binding within the pore was the primary interaction; transferring aa 37–45 did not confer sensitivity. In addition, a series of biophysical and structural investigations using the TM domain peptide favoured amantadine binding within the lumen, including the refined ssNMR structure of amantadine-bound M2.[79,126,143,144] Furthermore, the solution structure of a chimeric A/BM2 conductance domain construct with the TM domain