Determining the mechanism of action of lipophilic compounds by the development of a three-way equilibrium radioligand binding assay.

Darren Riddy[1], Sam Miller[2], Chris Langmead[3], Mark Dowling[1]. *[1]Novartis Institutes for BioMedical Research, Horsham, West Sussex, RH12 5AB, UK, [2]University of Leicester, Leicester, LE1 7RH, UK, [3]Heptares Therapeutics, Welwyn Garden City, Hertfordshire, AL7 3AX, UK*

Over the past decade there has been a growing interest in the G-protein coupled receptors (GPCR) for sphingosine-1-phosphate (S1P) as therapeutic targets amenable to pharmacological intervention. High affinity ligands at these GPCRs are general lipophilic in nature and in our experience exhibit poor solubility, low permeability and high non-specific binding. This makes performing comprehensive mechanism of actions studies difficult, as there is a resultant restriction in the useful working experimental concentration range of these ligands. To overcome this technical challenge, we adapted a previously published method based on the extended ternary complex model (ETCM) which quantifies the interaction of two orthosteric ligands with a putative allosteric compound at the same receptor (Lanzafame *et al*., 2006).

To validate this system we first used the Muscarinic M_2 receptor for which there are well characterised ligands described as either competitive (ipratropium, atropine) or allosteric (gallamine) (Burke, 1986) with respect to [^{3}H]-NMS. The pK_d for [^{3}H]-NMS determined from radioligand saturation binding studies was 9.54 $\pm$ 0.08. A family of atropine inhibition binding curves against a single concentration of [^{3}H]-NMS was performed in the presence of increasing concentrations of ipratropium or gallamine. The resulting IC_{50} curves were analysed with an extended version of the TCM (Lanzafame *et al*., 2006) to estimate the affinity (pK_i) and any co-operativity factor (α) of the unlabelled compounds. Using this model the estimated pK_i affinity values for atropine and ipratropium were 9.19 $\pm$ 0.20 and 8.05 $\pm$ 0.60 respectively. No meaningful α values could be obtained indicating that these two ligands share a competitive binding site with [^{3}H]-NMS. This is in contrast with gallamine, for which a pK_i value of 5.75 $\pm$ 0.69 was determined and an α value of 0.08 $\pm$ 0.09 for the interaction between gallamine and [^{3}H]-NMS and 0.06 $\pm$ 0.08 for gallamine and atropine. This confirms previous studies that gallamine binds to a common allosteric binding site on the M_2 receptor (Christopoulos, 2000).

Next we applied the model to the $S1P_3$ receptor using the radioligand agonist [^{3}H]-FTY720-P in combination with both unlabelled S1P and antagonists of interest. In order to apply the TCM model we sought first to establish that FTY720-P and S1P share a common binding site. Displacement binding of a range of [^{33}P]-S1P concentrations was performed using a full titration of FTY720-P. FTY720-P IC_{50} curves were proportionally rightward shifted with increasing [^{33}P]-S1P concentrations. Additionally the dissociation kinetics of [^{3}H]-FTY720-P in the presence of S1P or FTY720-P, resulted in $T_{½}$ values of 24.0 ± 8.4 and 24.2 ± 2.6 min^{-1}, implying these ligands bind to a common site. We have profiled a number of compounds using the three-way ligand binding assay, including 1-benzyl-*N*-(2,3-difluorobenzyl)-5-hydroxy-2-isopropyl-1*H*-indole-3-carboxamide (Allergan, US) and (*Z*)-*N,N'*-bis(4-chlorophenyl)-3,3-dimethyl-2-oxobutanehydrazonamide (TY-52156, Toa Eiyo, Japan), and present data which suggest these bind to allosteric sites distinct from the orthosteric ligands. All data presented is expressed as mean ± SD.