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What is pharmacological 'affinity'? Relevance to biased agonism and antagonism

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The differences between affinity measurements made in binding studies and those relevant to receptor function are described. There are theoretical and practical reasons for not utilizing binding data and, in terms of the quantification of signaling bias, it is unnecessary to do so. Finally, the allosteric control of ligand affinity through receptor–signaling protein interaction is discussed within the context of biased antagonism. In this regard, it is shown that both the bias and relative efficacy of a ligand are essential data for fully predicting biased effects *in vivo*.

> What we observe is not nature itself but nature exposed to our method of questioning. – Werner Heisenberg (1901–1976)

Binding: 'Langmuirian' affinity

Pharmacological affinity is a measure of the attraction a ligand has for a biological target. It can be quantified with an equilibrium dissociation constant, defined as the ratio of the rate that the ligand approaches the protein binding site (denoted k_1 in temporal units such as $s^{-1}M^{-1}$) and the rate that the bound ligand diffuses away from the protein binding site (denoted k_2 with units of s^{-1}). Thus the characteristic number used to quantify affinity is defined as k_2/k_1 and denoted K_A. The root model for calculation of K_A is the mass action equation, a relationship first derived by A.V. Hill [1] and made popular by the chemist Irving Langmuir 6 years later as the adsorption isotherm [2]. This equation (Equation 1) defines ρ_A, the fraction of available binding sites bound by a concentration of ligand [A]:

$$\rho_A = \frac{[A]B_{max}}{[A] + K_A} \quad [1]$$

where B_{max} is the maximal number of binding sites and K_A the equilibrium dissociation constant of the ligand–receptor complex. The application of this simple equation to the binding of ligands to proteins such as receptors has been of undeniable value to pharmacology, but the literal translation of the parameters obtained from experiments using this model should be made with caution. The originators of this equation defined its use and limitations. For example, as pointed out by Colquhoun [3], Hill was applying the equation to determine only if '... [an] equation of this type can satisfy all the observations, than to base any direct physical meaning on ... K_A'. In the case of Langmuir's application of the isotherm, he was primarily interested in the adsorption of gases to metal surfaces, a medium very different from receptor protein in that the binding surface is homogeneous and non-interactive with the ligand. The required homogeneity of the binding surface was highlighted by Langmuir himself who indicated that any heterogeneity (such as might be found for activated charcoal) could alter the interpretation of the parameters obtained; '.... but it is evident that [the] Equation, which appl[ies] to adsorption by plane surfaces, could not apply to adsorption by charcoal' [2]. The available binding sites on seven transmembrane receptors (7TMRs) most often do not satisfy either of the prerequisites of uniformity and non-interaction (*vide infra*).

Within the context of this discussion, K_A values derived from Equation 1 will be defined as 'Langmuirian binding' affinities. The binding of a radioligand to a 7TMR can be measured, and Equation 1 applied to the resulting data to yield a measure of K_A, but the question raised in this present discussion is – when it is appropriate for independent estimates through binding studies to be utilized as estimates of 'functional affinity'? The term functional affinity will be used to denote a number that can describe pharmacological activities of a ligand; these include the level of receptor occupancy for determination of the amount of activating complex for cellular signaling and also the receptor occupancy that causes antagonism of more efficacious agonists *in vivo*. As a preface to this discussion, it is important to define the species that drugs bind to, in this case, 7TMRs.

7TMRs as ensembles of allosteric proteins

7TMRs are the major means by which chemical signals are transmitted from the extracellular space to the cell cytosol. Their main function is to change their shape (conformation) in response to interactions with extracellular ligands and intracellular signaling proteins. There is a great deal of evidence to suggest that proteins such as these do not stay in static conformations but instead exist in ensembles of different conformations that interchange with the available free energy of the system [4–9]. In this sense, the

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binding surface for ligands is not homogeneous. This heterogeneity is further enhanced by the fact that ligand binding can selectively stabilize some conformations over others, and therefore the process of binding may change the relative proportions of these conformations [10]. If the ligand promotes a given conformation, which then reacts with a signaling protein or other cellular body, or even if the binding promotes stabilization of a different conformation, then the observed affinity of that ligand can be dependent upon these interactive processes. For a scheme where receptor R changes to another state R* upon binding of ligand A, the observed affinity of the ligand is not the molecular affinity for the R state but instead an affinity that depends upon the rate of the transformation to the R* state.

$$A + R \overset{K}{\rightleftarrows} AR \underset{\varphi}{\overset{\gamma}{\rightleftarrows}} AR* \quad [2]$$

Thus, the receptor 'isomerizes' [11] (becomes another thermodynamic species) through the processes controlled by γ and φ (the conformational state of the 7TMR is not confined to AR) and the observed affinity will be an amalgam value for K_A, very different from the static parameter defined by the adsorption isotherm [11]:

$$K_A = \frac{K}{1 + \frac{\gamma}{\varphi}} \quad [3]$$

In addition, a very important feature of 7TMRs is their allosteric nature. Extracellular ligands such as hormones or neurotransmitters stabilize conformations of the receptor that then promote binding and activation of cellular signaling proteins. Such changes in receptor conformation constitute the 'intrinsic efficacy' of the ligand and form the basis of direct agonism. This function is allosteric in that the binding of a ligand to the receptor alters its affinity for signaling proteins as they interact at other loci on the receptor; this flow of energy forms an allosteric vector [12] that is bidirectional in that the binding of a signaling protein to a receptor will concomitantly alter the affinity of the receptor for a ligand in other regions of the protein. This reciprocation of affinity is concisely defined by the allosteric binding model [13,14]:

$$\begin{array}{ccccc} A & + & BR & \overset{\alpha K_A}{\rightleftarrows} & ARB \\ & & \Updownarrow K_B & & \Updownarrow \alpha K_B \\ A & + & R & \underset{K_A}{\rightleftarrows} & AR \\ & & + & & + \\ & & B & & B \end{array} \quad [4]$$

Therefore, the affinity of the receptor R for the ligand A is modified by the interaction of the receptor with allosteric ligand B by the factor α, and the affinity of the receptor for ligand B is modified to the same degree (namely α) by the binding of ligand A. Under these circumstances the observed affinity of the ligand A for the receptor will depend both on the nature and concentration of the co-binding allosteric ligand by the expression:

$$K = \frac{K_A(1 + [B]/K_B)}{(1 + \alpha[B]/K_B)} \quad [5]$$

Because allostery is probe-dependent (i.e., every modulator will have a unique value of α for every co-binding ligand), Equation 5 shows how the functional affinity K can vary with both the amount and type of allosteric co-binding ligand. This probe-dependent effect has been demonstrated in numerous experimental situations; for example, the allosteric inhibitor aplaviroc of the receptor CCR5 [chemokine (C-C motif) receptor 5] produces widely divergent effects on the affinity of chemokines from a greater than 30-fold decrease in the affinity of the receptor for CCL3 [chemokine (C-C motif) ligand 3] to nearly no effect on the affinity of the chemokine CCL5 [15].

Several allosteric ligands produce effects on ligand receptor affinity through interaction at extracellular sites but there is no *a priori* reason to suppose that these actions cannot be mediated via the cytosol. For example, a series of CCR4 and CCR5 antagonists have been shown to produce allosteric blockade of chemokine receptors by binding to sites inside the cell [16]. Similarly, signaling proteins such as G proteins or β-arrestin will function as allosteric modulators of the receptor, and it follows that the affinity of the receptor for different ligands (e.g., agonists) can differ by varying α-values as different signaling proteins associate with the receptor. This effect is dramatically illustrated by the difference in the X-ray crystallographic structure of the β_2-adrenoceptor bound and not bound to a nanobody simulating a G protein [17,18]. Similarly, differences in the nature of the affinity changes with different signaling co-binding proteins are observed for ghrelin receptors in lipid discs – clear changes in the conformations of the receptors have been demonstrated following addition of G_q to nanodiscs [19]. Studies using exponential fluorescent lifetime decay analysis also show the creation of different conformations upon addition of β-arrestin [19]. κ-Opioid receptor SCAM (substituted cysteine accessibility method) studies indicate changes in conformation in 7TM domains 6 and 7 with binding of $G_{\alpha 16}$ and/or $G_{\alpha i2}$ G protein subunits, and this is reflected in an 18-fold change in affinity for the ligand salvanoran [20]. In general, there is abundant evidence to show that receptors can complex with membrane proteins to yield multiple affinity states, a condition Black and Shankley referred to as 'receptor distribution' [21]. These types of data underscore the impact of membrane components on agonist affinity for receptors.

Membrane components can alter antagonist affinity as well. For example, the membrane protein RAMP3 (receptor activity modifying protein [22]) produces dramatic effects on the blockade of human calcitonin receptor by the antagonist peptide AC66 [23]. The Schild regressions shown in Figure 1A,B show how cotransfection of RAMP3 produces a 10-fold decrease in the affinity of the antagonist AC66 in blocking the effects of amylin, but has no effect on the antagonism of human calcitonin; this illustrates both the allosteric control of antagonist affinity of a receptor for ligands by a membrane-bound species and also, the probe-dependence of that allosteric control.

Functional affinity

This discussion will define the term 'functional' affinity as being the K_A value – defining the strength of binding of a ligand as it forms a complex with the receptor to both induce a cellular response and also to interfere with the