Selectivity of pharmacological tools: implications for use in cell physiology.  
A Review in the Theme: Cell Signaling: Proteins, Pathways and Mechanisms

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Michel MC, Seifert R. Selectivity of pharmacological tools: implications for use in cell physiology. A Review in the Theme: Cell Signaling: Proteins, Pathways and Mechanisms. Am J Physiol Cell Physiol 308: C505–C520, 2015. First published January 28, 2015; doi:10.1152/ajpcell.00389.2014.—Pharmacological inhibitors are frequently used to identify the receptors, receptor subtypes, and associated signaling pathways involved in physiological cell responses. Based on the effects of such inhibitors conclusions are drawn about the involvement of their assumed target or lack thereof. While such inhibitors can be useful tools for a better physiological understanding, their uncritical use can lead to incorrect conclusions. This article reviews the concept of inhibitor selectivity and its implication for cell physiology. Specifically, we discuss the implications of using inhibitor vs. activator approaches, issues of direct vs. indirect pathway modulation, implications of inverse agonism and biased signaling, and those of orthosteric vs. allosteric, competitive vs. noncompetitive, and reversible vs. irreversible inhibition. Additional problems can result from inconsistent estimates of inhibitor potency and differences in potency between cell-free systems and intact cells. These concepts are illustrated by several examples of inhibitors displaying affinity for related but distinct targets or even unrelated targets. Of note, many of the issues being addressed are also applicable to genetic inhibition strategies. The main practical conclusion following from these concepts is that investigators should be critical in the choice of inhibitor, its concentrations, and its mode of application. When this advice is adhered to, small-molecule pharmacological inhibitors can be important experimental tools in the hand of physiologists.

amiloride; amiodarone; biased signaling; competitive antagonism; functional antagonism; inverse agonism; SQ22,536; SR 59,230A; selectivity; suramin; U 73,122

PHYSIOLOGICAL STUDIES often use small-molecule pharmacological inhibitors to explore the involvement of receptors and their signaling pathways in cellular or tissue responses to a given neurotransmitter, hormone, or local mediator. Specific inhibitors can be useful tools in this regard, but, if applied uncritically, they can result in erroneous conclusions and turn into weapons of mass deception. Therefore, the present article aims to critically review limitations of commonly used pharmacological inhibitors as applied to physiological studies, particularly at the cellular level. This is illustrated by several examples; however, these examples are just that and should not be mistaken for a comprehensive list of “problem compounds.”

Genetic Approaches, Antibodies, and Small Molecules

Three broad types of inhibitors are available as tools for physiological studies: genetic inhibitors, antibodies, and small molecules. Genetic inhibitors can be subgrouped into knockouts, knockdowns, and transfection with dominant-negative mutants. Knockouts can be constitutive or inducible and systemic or cell type specific; they are mostly applied to whole-animal studies with read-out parameters at the in vivo, isolated tissue, or isolated cell level. Knockdown approaches, also referred to as gene silencing, can be based on antisense constructs or, more recently, small interfering RNA and often are applied to isolated tissues or cells. An inherent advantage of these molecular approaches is potentially high target selectivity. However, they are not by definition target selective, and in particular antisense and small interfering RNA approaches require careful control based on missense or scrambled sequences (134). Moreover, knockout of one gene may lead to compensatory regulation of other gene products that funnel into the same phenotype (16, 185). This problem is most prominent with constitutive systemic knockout but may also occur with other molecular approaches. Moreover, such approaches may not be feasible in cases where an embryonic knockout deleteriously interferes with the development of the animal. Also, the phenotypic consequences of knockouts may depend on the rodent strain being used, particularly with regard to immunologic and behavioral parameters (153, 170, 171). Finally, it should be considered that knockout or knockdown of a protein interacting with a receptor may affect not only the function of that particular receptor but also the function of other proteins. An eminent example for this is arrestin, which...
plays a role not only in receptor signaling but also as a scaffolding protein involved in cell migration and cytoskeletal reorganization (141); hence, any effect of arrestin knockout or knockdown is not necessarily related to its role in receptor signaling.

One key limitation of genetic inhibitor approaches is technical problems such as poor transfection efficiency for some (most often highly differentiated) cell types. A second problem is a slow onset of action that is limited by the turnover rate of already-existing gene product. Concerns about gene dose-response effects also need to be considered, and in particular interpretation of data from knockdown approaches can be limited by a lack of knowledge of how much remaining expression of the target gene is necessary to yield a functional response (threshold effect). Thus genetic approaches in general have high potential for target selectivity but technically are more challenging than other inhibition approaches and require considerable time frames of pretreatment. Of note, most of these genetic approaches can be used not only to inhibit but also to activate a pathway of interest (see next section for a general discussion of inhibitor vs. activator approaches).

Antibodies could theoretically be alternative tools to specifically inhibit receptors in living cells, but their use has three potential problems. First, most antibodies against G protein-coupled receptors (GPCRs) (2, 9, 14, 18, 31–33, 139, 160, 190, 208) or ligand-gated ion channels (61, 100, 145) lack selectivity for their target receptors. While this problem has long been underestimated because of inappropriate validation, it is now recognized as being widespread, and among commercially available GPCR antibodies lack of selectivity is considered the rule rather than the exception (111). Second, some receptor antibodies behave as receptor antagonists, whereas others, often autoantibodies, behave as agonists (118, 140, 198); for most commercially available antibodies their agonist vs. antagonist properties have been insufficiently explored and their use is largely limited to immunoblotting and immunochemistry rather than as functional inhibitors. Third, unless specific measures are taken, only antibodies directed against epitopes in the extracellular domain of the target protein can be used in studies with intact cells (14). If cell permeabilization approaches are used to allow antibody access to intracellular targets, such permeabilization per se may interfere with cell function. Therefore, the focus of this article is on small molecules as pharmacological inhibitors of receptors and their signal transduction pathways.

**Inhibitor vs. Activator Approaches**

The involvement of a specific pathway in a given response is preferentially tested by selectively inhibiting that pathway. This can involve genetic or antibody-based approaches or small-molecule inhibitors. However, sometimes it is not feasible to directly test for inhibition, for instance, because no suitable inhibitor exists. In such situations, conclusions are sometimes drawn on the basis of experiments where factor A activates pathway X and cellular response Y and a distinct factor B also activates pathway X and response Y; this is taken as evidence that Y is mediated by X. While this approach sometimes represents the best possible evidence based on available tools, the results need to be interpreted cautiously as they generate only circumstantial evidence and can be misleading. For instance, muscarinic or bradykinin receptors in urinary bladder smooth muscle can stimulate phospholipid (PL)C. PLC activation is known to elevate intracellular Ca\(^{2+}\) levels, and elevation of intracellular Ca\(^{2+}\) is known to elicit smooth muscle contraction. Nevertheless, it was found in multiple species that an inhibitor of PLC in a concentration at which it fully suppresses inositol phosphate formation did not attenuate urinary bladder contraction stimulated by muscarinic receptor agonists (172, 173) or bradykinin (165). Similarly, β-adrenoceptor (AR) stimulation promotes cyclic AMP (cAMP) formation and causes smooth muscle relaxation; both responses are mimicked by the direct adenylyl cyclase stimulator forskolin. Nevertheless, in smooth muscle of, e.g., blood vessels (63), airways (115, 191), and the urinary bladder (66, 193) β-AR-mediated relaxation largely occurs in a cAMP-independent manner and rather involves activation of K\(^{+}\) channels. Along the same lines, membrane-permeant cAMP analogs in neutrophils and β2-AR agonists inhibit superoxide formation, indicating an involvement of cAMP in such inhibition. However, the potencies and efficacies of β2-AR agonists at increasing cAMP and inhibiting superoxide formation differ considerably (25). Therefore, the identification of pathways involved in a physiological response should preferentially be based on inhibitor rather than activator approaches. If inhibitor approaches are not feasible, data based on activator approaches need to be interpreted cautiously. This general consideration applies not only to small molecules but also to genetic approaches.

Caution must also be applied in the interpretation of data based on inhibitor approaches if the chosen inhibitor does not act on the pathway under investigation directly but by physiological antagonism. A classic example of physiological antagonism is the observation that stimulation of β-ARs increases frequency and strength of cardiomyocyte contraction and stimulation of muscarinic receptors inhibits such contraction (23). Nevertheless, this obviously does not imply that β-AR agonists act via muscarinic receptors or that muscarinic receptor agonists are β-AR antagonists; rather, both act on a shared downstream signaling event.

**Direct vs. Indirect Pathway Modulation**

The signal transduction pathways from receptor activation to cellular response are complex, typically involving multiple steps and branching points. Such complexity has a number of important implications for the use of inhibitors. This can be illustrated using β-AR as an example. The canonical signaling pathway of β-AR is coupling to a G\(_{16}\) protein, leading to activation of an adenylyl cyclase and formation of cAMP, which in turn activates cAMP-dependent protein kinase (PKA). While this pathway is present and active in each β-AR-expressing cell, it is not the only pathway leading to cellular responses. Thus at least some β-AR subtypes can couple not only to G\(_{16}\) but also to G\(_{1}\) proteins (46, 184, 200), the latter leading to inhibition of adenylyl cyclase and potentially to activation of some types of K\(^{+}\) channels (110). Moreover, they can couple to G protein-independent responses mediated by β-arrestin (60, 159) and/or to some (other) types of K\(^{+}\) channels and to Ca\(^{2+}\) channels (68). Finally, cAMP can activate not only PKA but also the exchange protein activated by cAMP, Epac, which in some cases elicits a response different from PKA activation (168). While each of these branches of
β-AR signaling can be inhibited, such inhibition obviously allows conclusions only about that specific pathway and not about β-AR signaling in general. Moreover, all of these responses may contribute to a cellular response such as changes in membrane potential and myosin light chain phosphorylation leading to smooth muscle relaxation. Therefore, interpretation of the effect of an inhibitor always depends on the specific step in the signaling cascade of a receptor that is inhibited by that inhibitor.

**Inverse Agonism and Biased/Ligand-Directed Signaling**

For a long time it had been assumed that pathway inhibitors acting at the level of the receptor, i.e., receptor antagonists, are without effect in the absence of endogenous or exogenous agonist. Meanwhile, it is generally accepted that this applies to only a very small number of antagonists for most model systems and/or to a small number of models (45, 180); such antagonists now are more specifically referred to as neutral antagonists. It has now been realized that GPCRs exist in multiple conformations, and that agonists and antagonists shift the equilibrium between such states. Thus many antagonists actually are inverse agonists, i.e., inhibit basal receptor function in the absence of agonist as shown for instance for AT1 angiotensin II receptors (142), α1-AR subtypes (45, 84), β2-AR (39), muscarinic receptor subtypes (151), and histamine receptor subtypes (144). The extent of such inhibition depends on the degree of spontaneous activity, which differs between receptors and the cells in which they are expressed. Specifically, systems with high levels of receptor expression are likely to exhibit constitutive activity and hence detectable inverse agonism by many antagonists, and the same applies to some naturally occurring constitutively active receptor variants (45, 156, 161, 180, 182, 183, 187). The difference between inverse agonists and neutral antagonists may be subtle in in vitro studies, as both block the effects of endogenous agonists, but more visible in vitro when endogenous agonist is absent. Although biased agonism is presently largely shown in in vitro systems, examples convincingly demonstrating biased agonism in vivo are beginning to emerge (59, 73, 154). In several cases, antagonists including inverse agonists for some responses can be partial or even full agonists for other responses mediated by the same receptor if they exhibit biased agonism. Of note, the term “biased agonism” always implies comparison to a reference system or reference compound. For example, in system bias a receptor preferentially couples to one pathway in one cell and to another pathway in another cell; ligand bias exists relative to a reference ligand, which most often is the endogenous ligand.

This phenomenon can be illustrated by the β-AR ligand SR 59,230A. In cellular signaling studies SR 59,230A was a neutral antagonist for cAMP formation in 3T3-F442A murine adipocytes and cells transfected with the mouse β2-AR at a low receptor density, a partial agonist for cAMP formation in transfected cells expressing this receptor at a high density, and an (almost) full agonist for extracellular acidification rate in transfected cells (98, 167). Moreover, when cells expressing different β2-AR densities were compared, the degree of agonism increased with that of receptor level for cAMP formation but not for extracellular acidification rate (98, 167). The physiological relevance of partial agonism for some but not other β-AR-mediated responses within a given transfected cell has been demonstrated by the observation that SR 59,230A behaves as a partial agonist for the relaxation of smooth muscle in mouse ileum (167) and in rat and human urinary bladder (67) (Fig. 1).

The problem of inverse and biased agonism gets even more complex when it is considered that a wide range of factors including sex, age, disease, and treatments can affect the expression and function of receptors and of molecules in their signal transduction pathways. The potential impact of changes in receptor number on antagonist vs. agonist and on biased agonism properties is illustrated by the above example of SR 59,230A. Another illustration of this involves cardiomyocytes obtained from animals or patients with congestive heart failure; these exhibit desensitization and downregulation of β1-AR, activated receptors (89). Most likely, this is a phenomenon applying to all GPCRs with the exception of rhodopsin, for which only one agonist exists, light. Although biased agonism is presently largely shown in in vitro systems, examples convincingly demonstrating biased agonism in vivo are beginning to emerge (59, 73, 154). In several cases, antagonists including inverse agonists for some responses can be partial or even full agonists for other responses mediated by the same receptor if they exhibit biased agonism. Of note, the term “biased agonism” always implies comparison to a reference system or reference compound. For example, in system bias a receptor preferentially couples to one pathway in one cell and to another pathway in another cell; ligand bias exists relative to a reference ligand, which most often is the endogenous ligand.

**Fig. 1.** Effects of the antagonist/biased agonist SR 59,230A on 50 mM KCl-induced tension of human urinary bladder strips. Peak KCl responses were determined after an exposure of bladder strips to 10 μM SR 59,230A for 10 min. Each data point represents a sample from 1 human bladder. Horizontal lines indicate group means with standard deviations; P = 0.0465 in a paired t-test. Drawn based on data shown in Frazier et al. (67).
little if any alteration of β₂-AR, a downregulation of Gₛ, and upregulation of Gᵢ, β-arrestin-1, and GPCR kinases (22). Given that β₂- and β₃-AR can couple to both Gₛ and Gᵢ proteins (46, 184, 200) and that some ligands at these receptors are biased for either of these G proteins or β-arrestin, it becomes obvious that a disease state may alter the observed biased agonism properties of receptor ligands. We have recently proposed to term this phenomenon “dynamic bias” (138).

A practical consequence of inverse and biased agonism is that a presumed antagonist may lower or increase baseline for a given physiological response, respectively, and this shifted baseline may affect interpretation of the results with the agonist under investigation. For example, an agonist may stimulate cAMP formation to 150% of control, whereas an inverse agonist may reduce it to 50% of control; if the agonist + inverse agonist combination yields 75% of the original control, does that mean that the inverse agonist/antagonist inhibited the response or that the agonist, relative to the lowered baseline, still increased the response by 50%? Such data are not easy to interpret, but acknowledging such complexities is better than ignoring the problem and simply claiming antagonism in the interpretation. Moreover, noncompetitive interaction is observed if one of the ligands binds in an irreversible manner; for practical purposes agents with a very slow dissociation rate can also behave as irreversible, for instance when the dissociation \( t_{1/2} \) is greater than the duration of the assay. Of note, functional or physiological antagonism is never competitive, as the two ligands bind to different target molecules, for instance with β-AR and muscarinic receptor agonists increasing and decreasing heart rate (23). Moreover, apparent noncompetitive antagonism does not necessarily result from ligand interaction with an allosteric site but can also be due to nonequilibrium conditions of the system (108). The classification of inhibitors into competitive and noncompetitive agents has a number of practical implications.

The occupation of a receptor or other target protein by a ligand can be estimated from the equation

\[
\text{fractional occupancy} = \frac{[L]}{[L] + K_d}
\]

where \([L]\) and \(K_d\) are the concentration of the ligand and its dissociation constant, respectively. From this equation it appears that \([L]\) typically needs to be \(\sim\)100 times \(K_d\) to yield full occupation of a receptor. For antagonists, the degree of occupation typically equals that of inhibition of a receptor. In contrast, agonists sometimes only need to occupy a small fraction of a receptor to yield maximum activation; this can happen under circumstances of nonlinear occupation-response coupling, more commonly known as “receptor reserve” or as presence of “spare receptors.” For example, only \(\sim\)10% receptor occupancy is required for isoprenaline to cause an almost maximum positive inotropic effect in rat heart (24); in such cases, the agonist concentration causing a half-maximal response typically is much lower than its affinity as assessed by the \(K_d\), which is the concentration required to occupy half of the receptors. A receptor reserve is often observed if receptor expression is large, for instance in transfected cells (192), or when cells have a very efficient signal transduction machinery. The latter also explains why the degree of receptor reserve within one cell may differ between concomitant cellular responses, typically being largest for the most distal response, i.e., when most amplification steps have been involved along the way.

Although the initial binding of irreversible ligands to their target can be competitive, their lack of dissociation implies that they cannot act competively. Nevertheless, irreversible inhibitors can exhibit subtype selectivity, but the target selectivity of irreversible inhibitors depends strongly on assay conditions. An example is chloroethylclonidine, an α-AR ligand that has been instrumental in the early definition of α₁-AR subtypes, where α₁B- and α₁L-ARs were considered chloroethylclonidine sensitive and insensitive, respectively (80). However, similar to many irreversible compounds, the alkylation effect of chloroethylclonidine is time and temperature dependent (136). Thus with longer incubation times or higher temperature conditions, irreversible inhibition may be observed. However, these are not competitive in nature, and it is important to consider the time of incubation when deriving conclusions from experiments with irreversible inhibitors.
chloroethylclonidine can also fully inactivate $\alpha_{1A}$-AR and, with even longer incubation, also subtypes of $\alpha_{2}$-AR. This renders the interpretation of data obtained with irreversible agents difficult. Therefore, the interpretation of data obtained with irreversible inhibitors hinges on specific aspects of the assay being employed, including incubation time and temperature.

**Analytical Methods to Determine Inhibitor Potency and Affinity**

Many antagonists interact competitively with a receptor, i.e., the degree of inhibition depends on the ratio between antagonist and endogenous agonist concentrations. This means that the concentration of antagonist required to achieve a 50% inhibition of a response to a given agonist, the IC$_{50}$, is not a constant but depends on the agonist concentration. Therefore, pharmacologists have developed a number of methods to experimentally determine the inhibition constant of an antagonist, which, depending on the assessment method being used, can be reported as pK$_B$, apparent pA$_2$, or $K_i$ value. The most informative of these approaches is the Schild plot (8), and the affinity estimate derived from it is termed the pK$_B$ value (152). Importantly, a Schild plot not only allows functional estimation of antagonist affinity but also is sensitive in picking up the presence of nonequilibrium states in the assay, which are indicated by a nonlinear Schild plot or one with a slope different from unity. The presence of nonequilibrium precludes conclusions on antagonist affinity. Sources of nonequilibrium in an assay include the presence of mechanisms affecting the actual drug concentration. These can include transporters such as neuronal and nonneuronal transporters for biogenic amines or ligand-degrading enzymes including monoamine oxidase for catecholamines, acetylcholinesterase for acetylcholine, or various peptidases for peptides. Other sources of nonequilibrium are poor chemical stability of agonists or antagonists, including sensitivity to oxidation (82). Even if agonist and antagonist concentrations in the assay remain constant at their nominal level, nonequilibrium is possible if the response to an agonist involves more than one receptor subtype and the antagonist has differential affinity for them or when the interaction between agonist and antagonist is noncompetitive. For all of these reasons, the Schild plot is considered the most reliable way to obtain antagonist affinity estimates in functional assays.

When it is not feasible to test multiple antagonist concentrations, an apparent pA$_2$ value can be determined based on the ability of a single antagonist concentration to shift the concentration-response curve of the agonist. While determination of an apparent pA$_2$ value is less cumbersome than testing multiple antagonist concentrations under such conditions, it relies on the assumption that the interaction between agonist and antagonist is competitive and that the experiment has been performed under equilibrium conditions.

An alternative approach for determining antagonist inhibitor potency has been derived from enzymology. In this approach a concentration-response curve is generated for an antagonist and the resulting IC$_{50}$ is converted to $K_i$ with the Cheng-Prusoff equation:

$$K_i = \text{IC}_{50} / \left[ (\text{agonist})/\text{EC}_{50} \text{ agonist} \right] + 1$$

where [agonist] is the agonist concentration in the assay and EC$_{50}$ the agonist concentration causing a half-maximal response (37). Similar to apparent pA$_2$ values, the Cheng-Prusoff approach provides less mechanistic information on the type of inhibition and depends on the same assumption on the presence of equilibrium and the competitive nature of the interaction between antagonist and agonist. However, it is the method of choice for determining the affinity of a competing ligand in radioligand binding assays.

**Consistency of Affinity and Selectivity Estimates**

According to pharmacological theory, the affinity of a ligand for a given receptor is a constant (101); it is typically expressed as $K_a$, $K_i$, or logarithm thereof or as a pA$_2$ value (152). While it is acknowledged that affinity estimates may vary to a certain degree, particularly in complex assay systems such as multicellular preparations with penetration barriers, the experimentally observed variation of receptor affinity estimates can be much greater than generally assumed. Binding studies in membrane preparations are the most often applied method to estimate ligand affinity. Because of the simplicity of the assay it could be expected that results from such studies exhibit limited variability across laboratories. For instance, affinity estimates of mepyramine for H$_1$ histamine receptors are quite consistent across laboratories (179, 187). However, for other receptors this is not the case. For instance, a review of various subtype-selective antagonists at cloned $\alpha_{1A}$-AR subtypes found that reported affinities exhibited a range of 1.5 log units for almost every compound at each subtype (135); given that most of these compounds exhibited a subtype selectivity of $<2$ log units for one $\alpha_{1A}$-AR compared with another within a given study, this was a worrisome finding. More recently, a similar analysis was carried out for eight different antagonists at angiotensin II type 1 receptors (133); in that review, based on up to 63 studies per drug, reported affinity values for almost every one exhibited a range of $>2$ log units, in one case even $\sim4$ log units (Fig. 2). Affinity estimates based on functional studies with signaling responses in isolated cells or for inhibition of vascular smooth muscle contraction yielded a similarly

![Fig. 2. Reported antagonist affinities in radioligand binding studies with AT$_1$ angiotensin II receptors. Each data point represents 1 study; horizontal lines indicate group means with standard deviations. Taken with permission from Michel et al. (133).](http://ajpcell.physiology.org/)
somber picture. However, within a given study parallel affinity estimates from binding and signaling and/or contraction studies typically yielded very similar estimates for a given drug. Such findings urge great caution in the uncritical use of affinity estimates from a single study when deriving conclusions based on a single subtype-selective inhibitor. Rank order of potency of multiple subtype-selective compounds within a given study typically is a more robust approach to define receptor subtypes involved in a given response.

Moreover, it should be considered that subtle differences in amino acid sequence of a receptor between species may yield substantial differences in affinity estimates for some compounds. For example, the affinity estimate for the angiotensin type 1 receptor antagonists losartan and valsartan is 1.5 log units lower at the canine receptor compared with that of several other species (49) (dog data have been excluded from Fig. 2 for this reason). Species differences in affinity estimates of 1 log unit or more have also been reported, e.g., various ligands at all histamine receptor subtypes (187) or at mouse vs. human formyl peptide receptors (207), for porcine vs. human V2 vasopressin receptors (93), or for BRL 37,344 (122, 150), CGP 12,177 (122), and L 748,337 (195, 196) at rat vs. human β2-AR; for L 748,337 an affinity difference between rat or rhesus monkey of ~100-fold was reported (29).

A good example of how species difference in drug affinity can have major consequences for the interpretation of experimental data comes from the α2-AR field, where the rat and bovine subtypes differ considerably in their pharmacological profile from their porcine and human orthologs, specifically with regard to their affinity for the antagonists rauwolscine and yohimbine (123, 155). This species difference in affinity for important antagonists can largely be explained by a serine to cysteine single amino acid switch in transmembrane region 5, amino acid position 5.43 (116). Lack of recognition of this species difference had originally led to the proposal of a fourth mammalian α2-AR subtype, the α2D-AR, as rauwolscine and yohimbine are considered as the prototypical antagonists to identify α2-AR (27). Thus the selectivity of an inhibitor in one species does not necessarily predict similar usefulness in another species, at least not with regard to affinity.

### Cell Permeability: Differential Potency in Cell-Free Systems and Intact Cells

To act on an intracellular signal transduction pathway, an inhibitor must first pass the cell membrane. A serious limitation of such studies is that nominal concentrations in the extracellular buffer do not necessarily predict those at the target, i.e., inside the cell. Rather, the actual intracellular concentrations are unknown in most cases, as they have not been determined by HPLC or, even more accurate, HPLC mass spectrometry-based methods. The ability of an inhibitor to pass the cell membrane is primarily determined by its lipophilicity. If lipophilicity is low, a concentration gradient will develop, i.e., intracellular inhibitor concentrations will be markedly lower than those in the extracellular milieu. In practical terms this means that extracellular concentrations must markedly exceed affinity at the target protein to effectively inhibit the intracellular target. For example, 8-bromo or dibutylryl analogs of cyclic nucleotides are often administered extracellularly to mimic the intracellular effects of cAMP and cGMP; while they have increased lipophilicity and show better cell penetration than the endogenous nucleotides, their overall penetration remains poor so that typically millimolar concentrations are required despite a target affinity in the nanomolar range (174). Similar considerations apply to in vivo studies when the physiological response under investigation involves the central nervous system; access of inhibitors can be limited by their degree of penetration through the blood-brain barrier, which, similar to penetration into cells, is typically/often limited by lipophilicity of the inhibitor (Table 1).

Some inhibitors can be used without being lipophilic as they are substrates for transporters (36), which reduce any concentration gradients or even create reverse ones. However, more frequently the opposite happens, i.e., many cells express transporter molecules that actively extrude compounds that have passively permeated the cell membrane. The pattern of expression of such transporters varies across cells and within a cell or tissue can dynamically be regulated by various factors (147). For most cell types the nature and extent of expression of transporter molecules are not fully known. This implies that not only are extracellular concentrations of an inhibitor poor predictors of intracellular concentrations but, to make matters worse, even the ratio of intra- to extracellular concentration is not easily predicted.

Accordingly, the required concentration of a given inhibitor may be much higher in intact cells than in cell-free systems, and published in vitro inhibitor potency often provides only initial guidance on extracellular concentrations required to yield inhibition of intracellular targets. In very few if any models have intracellular inhibitor concentrations been determined upon extracellular administration, partly because analytical methods with sufficient sensitivity and specificity in most cases are not available. If multicellular preparations are used, e.g., tissue strips or in vivo preparations, additional penetration barriers may be involved, including the vascular endothelium or the blood-brain barrier. Of note, whether a given compound is substrate to a given transporter is typically not related to its mechanism of action but rather to its specific

<table>
<thead>
<tr>
<th>Compound</th>
<th>Brain/Plasma Concentration Total</th>
<th>Brain/Plasma Concentration Free</th>
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<tbody>
<tr>
<td>5-HMT</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>N-methyl-scopolamine</td>
<td>0.05</td>
<td>0.02</td>
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<tr>
<td>Oxybutynin</td>
<td>6.27</td>
<td>3.30</td>
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<tr>
<td>Scopolamine</td>
<td>3.42</td>
<td>1.90</td>
</tr>
<tr>
<td>Solifenacin</td>
<td>3.04</td>
<td>0.28</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>2.95</td>
<td>0.23</td>
</tr>
<tr>
<td>Trosiptum</td>
<td>0.03</td>
<td>0.01</td>
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</table>

Data are expressed as ratio of brain to plasma concentration [(ng/g)/(ng/ml)] for total concentrations and after correction for protein binding (free). Rats received an intravenous bolus dose of 1 mg/kg (solifenacin and trosiptum) or 0.3 mg/kg (all other compounds), and measurements were made 1 h later. These data illustrate how multiple antagonists, all suitable for blocking peripheral receptors, are of different value when attempting to study central nervous system-mediated effects, as free concentration in the brain ranges from 1% to 330% of that in plasma. They further illustrate how the presence of protein in cell-free assays can differentially affect free drug concentrations. 5-HMT, 5-hydroxy-methyl-tolterodine. Adapted with permission from Callegari et al. (28).
chemical properties. For instance, this varies greatly within the drug class of clinically used muscarinic receptor antagonists, leading to differences in brain penetration and clinical tolerability (28).

A striking example of problems that can arise with extracellular administration of inhibitors of intracellular targets are adenylyl cyclase inhibitors bearing a polyphosphate chain. 2',3'-O-methylthraniloyl-substituted nucleoside 5'-triphosphates are potent competitive inhibitors of adenylyl cyclases in membrane preparations, often with nanomolar affinity (177). However, because of their negative charge, these compounds cannot readily penetrate the plasma membrane. One solution to the problem is the intracellular application of inhibitors via glass electrodes (96). This setting can be applied for electrophysiological studies, but it is still unknown what the actual inhibitor concentration at the pharmacological target is. Moreover, even an inhibitor exhibiting high affinity for its target in a recombinant system does not preclude this compound from exhibiting off-target effects (95, 96). As an alternative strategy, negatively charged phosphate groups can be protected by lipophilic substituents so that nucleotide prodrugs can cross the plasma membrane (117). These prodrugs are then intracellularly metabolized to the pharmacologically active compounds. However, this approach is not without problems either, because it is difficult to assess how fast metabolism occurs and whether the removed protecting groups exhibit toxic effects.

To circumvent the permeability problems associated with polyphosphate chain-containing adenylyl cyclase inhibitors, purine derivatives without phosphate are often used in pharmacological studies aiming at enzyme inhibition in intact cells. A prototypical compound that is widely used for this purpose is SQ22,536 (177). Compared with polyphosphate chain-containing compounds, SQ22,536 is a much less potent adenylyl cyclase inhibitor. Most investigators have used SQ22,536 under the bona fide assumption that the compound actually inhibits adenylyl cyclase without ensuring that cAMP concentrations decrease in intact cells. However, recent studies have found SQ22,536 to cause partial inhibition only (17, 44, 186) or even to be ineffective at inhibiting adenylyl cyclase in intact cells (25). Moreover, SQ22,536 must be dissolved in organic solvents. This property, together with the low inhibitor potency, entails that sufficiently high SQ22,536 concentrations cannot be obtained in intact cells without suffering toxic effects of the organic solvent. The case of SQ22,536 highlights the necessity of controlling for solvent effects in inhibitor studies. Furthermore, it cannot be taken for granted that purine-containing compounds are selective for adenylyl cyclase. Specifically, SQ22,536 inhibits ERK signaling in neurons in an adenylyl cyclase-independent manner (55). The antiviral drug vidarabine is structurally closely related to SQ22,536 and was claimed to constitute a selective inhibitor of adenylyl cyclase 5 (99). However, a systematic follow-up study revealed that vidarabine is not selective for adenylyl cyclase 5 at all (20). Accordingly, claims that vidarabine can be used therapeutically as a selective adenylyl cyclase 5 inhibitor to treat heart failure are unsubstantiated (176). Therefore, investigators should apply great care when basing the choice of inhibitor concentrations for intracellular targets on potency determined in cell-free systems to avoid false negative data due to underdosing or false positive data due to off-target effects.

Selectivity Within and Outside Target Family

Subtypes of a receptor typically are evolutionarily related (69, 70), and the resulting sequence homology can translate into a similarity of their pharmacophores. For instance, rhodospin, β2-AR, and M2 muscarinic receptors exhibit qualitatively similar but qualitatively different binding pockets for orthosteric ligands (114). On a lower level, the three amino acids that serve as countereions for norepinephrine are conserved across all nine AR subtypes (189). Accordingly, many ligands exhibit similar affinity for all subtypes within a receptor (sub)families for instance, atropine for subtypes of muscarinic acetylcholine receptors (30); prazosin for subtypes of α1-AR (135), yohimbine for subtypes of α2-AR (at least in some species) (27), and SR 59,230 for subtypes of β-AR (34). Use of ligands binding with similar affinity to various subtypes of a receptor can be useful to initially assess whether a receptor (sub)families is involved in a given response, often as initial classification for the subsequent identification of a specific receptor subtype. However, often investigators are interested in selectively inhibiting one specific receptor subtype. Many compounds have been proposed to do so, but the conclusions were later shown to be premature. For example, the α1-AR antagonist prazosin binds with relatively high affinity to α2B-AR and α2C-AR but not to α2A-AR (27); while the affinity for these subtypes is much lower than that for α1-AR, the use of high prazosin concentrations can cause misleading conclusions. Similar examples include the β1/β2-AR antagonist CGP 12,177 acting on α1-AR (19) and the highly β1-selective antagonist CGP 20,712 binding to α1-AR and subtypes of α2-AR (56) in concentrations exceeding those required to bind to their primary target. As another example, 4-methylhistamine has been broadly used as a selective histamine H4R antagonist, but in reality this compound is a dual H1R/H4R agonist, and the uncritical use of this compound may have resulted in erroneous conclusions regarding involvement of the H1R in certain systems (179, 187).

Such cross-reactivity can also be observed with targets less closely related to the primary binding partner of a ligand. For example, various histamine receptor antagonists bind to muscarinic receptors (54), and the muscarinic receptor antagonist propiverine binds with similar affinity to all subtypes of muscarinic receptors but also to all three subtypes of α1-AR (206) and also inhibits L-type Ca2+ channels (205). Interestingly, metabolites of propiverine exhibited a differential ratio of affinity for muscarinic receptors vs. α1-AR and/or vs. L-type Ca2+ channels (Table 2). An example of an antagonist at one receptor also acting on an only distantly related receptor is H1 histamine receptor antagonists binding to neuropsieptide Y Y1 receptors (3). Similarly, some angiotensin II type 1 receptor antagonists, including telmisartan, can additionally bind to unrelated GPCRs, i.e., thromboxane A2 receptors, and to non-GPCR receptors, i.e., peroxisome proliferator-activated receptor-γ, and this may contribute to their in vivo activity profiles (133). As another striking example, a formyl peptide analog protected by a tertiary butyloxycarbonyl group is not only, as expected, a formyl peptide receptor antagonist but also an antagonist at the receptor for the lipid mediator leukotriene B4 (199).
1-phosphate receptors (6, 164), A1 adenosine and D2 dopamine P2X and P2Y purinergic receptors (1, 109) and sphingosine
century ago to treat infections with protozoa and worms, is (15, 71). However, this compound, originally introduced a
suramin can directly interfere with receptor/G protein coupling
estrogens (74, 137).

Other than the above two examples of propiverine and
telmisarten, there are many others where GPCR antagonists additionally bind to non-GPCR targets. For example, the α1-AR antagonist naftopidil also is an L-type Ca2+-channel inhibitor (86), and several β-AR antagonists were shown to reduce the influx of Na+ and Ca2+ into neurons by directly interacting with L-type Ca2+-channels (90, 94, 129, 130, 181) and Na+ channels (40, 203). Similarly, some first-generation H1 histamine receptor agonists can directly activate G proteins in mast cells (26), the neuroleptic olanzapine can act on the
mammalian target of rapamycin (169), and several opioid
receptor ligands can also act on neuronal serotonin transporters (13). Effects at other targets have also been reported for ligands at non-GPCR receptors. For example, several estrogens can inhibit the neuronal norepinephrine, dopamine, and serotonin transporters, and, against the initial hypothesis, this is unrelated to the presence of a catechol moiety in the A-ring of some estrogens (74, 137).

Ligands directly affecting G protein-mediated signaling in many cases can also have off-target effects. For example, suramin can directly interfere with receptor/G protein coupling (15, 71). However, this compound, originally introduced a century ago to treat infections with protozoa and worms, is highly promiscuous. Thus suramin binds to some subtypes of P2X and P2Y purinergic receptors (1, 109) and sphingosine 1-phosphate receptors (6, 164), A2 adenosine and D2 dopamine receptors (15). Suramin also activates intracellular ryanodine receptors (202), blocks connexin 43 hemichannel permeability (38), and inhibits telomerase (57) and norovirus RNA-dependent RNA-polymerases (126). Similarly, NF449 was originally identified as a direct inhibitor of the G3, G4 protein with selectivity over G1 and G4 (88); however, later studies identified this compound to have much higher affinity for P2X1 receptors, actually exhibiting considerable selectivity for this compared with P2X2, P2Y1, and P2Y2 receptors (21, 104). NSC23766 is frequently used as a Rac1 inhibitor but also binds to muscarinic receptors (121).

At the level of G protein-activated signaling molecules, the diterpene forskolin is widely used as an activator of membra-
nous adenyl cyclases 1–8 (177); however, such diterpenes also bind to ion channels and glucose transporters (103), an effect almost always neglected in the interpretation of results obtained with forskolin. Similarly, U 73,122 is probably the most frequently used inhibitor to test the involvement of PLC in cell and tissue responses to agonists of Gαi-coupled rece-
tors. To obtain full inhibition of inositol phosphate formation in intact cells or tissue slice, a concentration of 10 μM is required (113). However, in such concentrations U 73,122 can also have PLC-independent effects including inhibition of platelet function (124) and direct binding to H1 histamine receptors (97). Even more worrisome are PLC-independent effects on targets that, similarly to PLC, are involved in the regulation of intracellular Ca2+ concentrations. These include inhibition of ion fluxes through some types of Ca2+-channels (125, 197) and K+ channels (41), activation of other ion channels (143, 149), inositol phosphate-independent release of Ca2+ from internal stores (143), enhancement of inositol phosphate effects on Ca2+ release (143), and inhibition of the sarcoplasmatic Ca2+-pump (127). The analog U 73,343 can be used as negative control for many nonspecific effects, but some PLC-independent effects of U 73,122 are shared by U 73,343 (41, 149). Despite these serious limitations, U 73,122 remains more useful than some other PLC inhibitors, particularly when used in combination with U 73,343 (65).

A class of enzyme inhibitors that has been notorious for lack of selectivity is protein kinase inhibitors, particularly those that act at the ATP binding site of protein kinases; this is not surprising, as the ATP binding site is well conserved across protein kinases. For instance, one group of investigators has systematically screened 42 protein kinase inhibitors in common use against a broad panel of kinases (11, 47). In this analysis KT 5720, rotterlin, and quercetin inhibited many protein kinases, sometimes with greater potency than that at their presumed targets. While several bisindolylmaleimides, H89, HA1077, and Y 27632, were more selective inhibitors, they still inhibited two or more protein kinases with similar potency. These findings were expanded in a more recent study that comprehensively tested 72 kinase inhibitors against 442 protein kinases that cover >80% of the human catalytic protein kinase (48). While such screening studies have identified a number of compounds with good selectivity for targeted vs. other kinases, these results show that the specificities of protein kinase inhibitors cannot be assessed simply by studying their effect on kinases that are closely related in primary structure. Of note, some kinase inhibitors may bind to targets outside the kinase family; for example, the protein kinase C inhibitors chelerythrine and bisindolylmaleimide I (also known as GF 103,203X) also directly block delayed-rectifier K+ channels (81) and the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY294002 potently blocks voltage-operated K+ channels (53).

Ion channel inhibitors also can exhibit selectivity problems as related to other types of ion channels or to non-ion channel targets. For instance, flecainide, an antiarrhythmic drug primarily inhibiting Na+ channels in the plasma membrane, can inhibit intracellular Ca2+-channels such as the ryanodine receptor (128). Similarly, amiodarone, a frequently used antiarrhythmic drug also inhibiting Na+ channels, can also block several types of K+ and Ca2+-channels and inhibit α- and β-ARs (83, 157) and additionally is a receptor-independent direct G protein activator (79). The frequently used Ca2+-channel inhibitors diltiazem and verapamil additionally are α- and/or β-AR antagonists (62, 146), and this apparently even affects their clinical profile (131).

Selectivity problems with transporter inhibitors have long been known from the group of monoamine uptake inhibitors.

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**Table 2. Differential affinity/potency of propiverine and some of its metabolites to muscarinic receptors, α1-AR, and L-type Ca2+ channels**

<table>
<thead>
<tr>
<th></th>
<th>α1-AR, log Ki</th>
<th>L-Type Ca2+ Channel, log IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propiverine</td>
<td>6.39</td>
<td>5.49</td>
</tr>
<tr>
<td>M-5</td>
<td>4.50</td>
<td>&lt;4</td>
</tr>
<tr>
<td>M-6</td>
<td>7.03</td>
<td>&gt;4</td>
</tr>
<tr>
<td>M-14</td>
<td>5.94</td>
<td>4.72</td>
</tr>
</tbody>
</table>

Data are based on radioligand binding studies at M1 muscarinic receptors and α1A-adrenoceptor (AR) with similar affinities at other subtypes of these receptors as reported by Wuest et al. (205, 206). Data on L-type Ca2+ channels are based on electrophysiological recordings in human urinary bladder smooth muscle cells as reported by Wuest et al. (204).
from the class of tricyclic antidepressants that directly bind to muscarinic receptors (54), \( \alpha_1 \)-AR (54, 75), and/or histamine receptors (7). Many of them also can bind, albeit with lower potency, to human organic cation transporters (77, 78). Even the so-called selective neuronal serotonin uptake inhibitors are not necessarily that selective, as fluoxetine can directly inhibit \( \text{Na}^+/\text{H}^+ \) channels (158). A final example from the group of transporter inhibitors is amiloride. This compound was originally introduced as a \( \text{Na}^+/\text{H}^+ \)-antiporter inhibitor (43) but has proven to be extraordinarily promiscuous. It also binds to various types of ARs, particularly \( \alpha_2 \)-AR (91, 92, 119), apparently by acting on an allosteric site of these receptors (120), and to \( \text{I}_2 \) imidazoline binding sites (52, 132). It also enhances atrial natriuretic factor binding to its receptor (50). Moreover, amiloride can interact with G proteins to interfere with adenyl cyclase inhibition (5). Recently, an amiloride binding pocket has been identified in GPCRs (76), which is identical to the allosteric \( \text{Na}^+ \) binding pocket in these receptors (188).

Bacterial and insect toxins, beyond the scope of this review, can be selective tools with respect to their mechanism of action (162), but this is not necessarily the case. For example, the wasp venom mastoparan was shown to be a GPCR-independent G protein activator (85) but unfortunately has multiple other effects including those on membrane fluidity, cell integrity, and other enzymes such as nucleoside diphosphokinases (102, 112), rendering interpretation of toxin effects in intact cells difficult.

To make matters worse, many compounds exist that are purported as inhibitors of various pathways—but aren’t. Rather, because of their chemical reactivity they interfere with...
multiple assays (rather than pathways) used to explore such pathways and therefore are referred to as pan-assay interfering substances (PAINS) (10). These include some 400 structural classes, but more than half of them fall into 16 recognizable categories (Fig. 3). Among these, toxoflavins and polyhydroxylated natural phytochemicals such as curcumin, epigallocatechin gallate, genistein, and resveratrol have proven particularly promiscuous. Nevertheless, genistein is frequently used as a tyrosine kinase inhibitor, but because of its PAINS properties results obtained with this compound basically cannot be interpreted.

**Role of the Right Concentration, Selectivity Is Relative**

The Renaissance physician Philippus Aureolus Theophrastus Bombastus von Hohenheim, also known as Paracelsus (1493–1541), already stated, “All things are poison, and nothing is without poison; only the dose permits something not to be poisonous.” Although half a millennium old, this remains the most important statement ever made about selectivity. Translated to pharmacological inhibitors, this means that we cannot assume that a compound will be equally informative as inhibitor in any concentration; if the chosen concentration is too low, incomplete inhibition will result, potentially leading to underestimation of the role of a given pathway and false negative results. On the other hand, if a too-high concentration is used, the compound may also inhibit other pathways and lead to false positive results. Either is equally deleterious to sound scientific conclusions.

This principle is illustrated by examples from the field of β-AR antagonists with selectivity for β1- over β2-AR, the moderately selective clinically used atenolol and the highly selective experimental compound CGP 20,712 (Fig. 4). In this example, atenolol exhibits ~20-fold selectivity for β1- over β2-AR (K_i of 388 and 8,140 nM, respectively). When full concentration-response curves are generated for this antagonist, its potency can be useful for identification of the β-AR subtype involved (assuming there are no β3-ARs in this preparation). If only a single concentration of 300 nM atenolol is used, there is hardly any occupancy of β2-ARs but less than half of the β1-ARs are occupied; if a higher concentration of 3 μM atenolol is used, occupation of β1-ARs is ~90% but almost 30% of β2-ARs are also occupied; at a concentration of 100 μM more than 90% of both subtypes would be occupied, making it basically a useless concentration for the identification of the subtype involved. A different situation arises when CGP 20,712, with ~1,000-fold selectivity for β1- over β2-AR, is used (K_i of 4.7 and 4,040 nM, respectively). At least some concentrations of this compound, e.g., 200 nM, will yield >90% occupancy of β1-AR but <10% occupancy of β2-AR; nonetheless, testing of multiple concentrations remains advisable, as different absolute affinities in the preparation under investigation may yield less or more occupancy of one of the subtypes.

**Practical Considerations**

From the above it is obvious that the choice of a suitable inhibitor for a receptor or signaling pathway of interest is far from trivial. Websites and catalogs of many fine chemical suppliers list affinities for a range of agents they are offering. However, the selection of references on which these are based may be biased, as it is attractive to make a compound look selective. We strongly recommend that whenever a researcher wishes to use a pharmacological tool in order to answer a physiological question, a thorough and comprehensive review of the primary literature be conducted. In many cases, claims about selectivity of a compound propagate almost automatically and as wishful thinking as soon as a critical number of publications has been reached. We also strongly recommend including, wherever possible, several structurally distinct compounds into the analysis, reducing the risk of erroneous data interpretation due to unexpected off-target effects. Moreover, it should be good practice to conduct concentration-response studies with a pharmacological tool and not only to use a fixed single concentration; 10 μM appears particularly popular among researchers because it is (often wrongly) assumed to be saturating for most targets without affecting off-targets. Furthermore, whenever possible, control experiments with related targets such as related receptor or kinase subtypes should be conducted, and in some cases inactive control compounds are available and should be examined. Last but not least, very simple control experiments such as cytotoxicity assay should be conducted to exclude nonspecific compound effects.

A good database for initial information on pharmacological tools is [www.guidetopharmacology.org](http://www.guidetopharmacology.org), which has been cre-

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**Fig. 4. Simulation of occupancy of β1- and β2-adrenoceptors by 2 subtype-selective antagonists, the moderately selective clinically used atenolol and the highly selective experimental compound CGP 20,712. Occupancy was simulated with GraphPad Prism based on affinity data from Hoffmann et al. (87).**
ated by the International Union of Basic and Clinical Pharmacology and the British Pharmacological Society based on the input of a wide range of experts. It is regularly updated and provides information on various receptors, ion channels, transporters, kinases and other enzymes, and their inhibitors. Of note, even if a given inhibitor compound is selective for its target, this does not mean that the preparation of the chosen supplier is; for example, some commercial preparations of the PKA inhibitors Rp-cAMPS were found to be contaminated with biologically active adenosine concentrations (148). More recently, some commercially available preparations of sphingosine 1-phosphate were found to contain contaminations in sufficiently high concentrations to obscure the effects under investigation (64).

**General Conclusions**

From the above it is clear that no experiment can have a better quality than that of the tools being used. Interpretation of receptor or signaling pathway inhibitor data is far too trivial, regardless of whether small molecules, antibodies, or genetic approaches are used. However, this should not discourage the use of inhibitors in the elucidation of molecular mechanisms controlling cell function. Rather, we advise investigators to always carefully check available information on the inhibitors to be used. Where possible, it is advisable to use multiple inhibitors of a given receptor or pathway of an inhibitor to increase the robustness of the data. Similarly, wherever possible, full concentration-response curves should be generated for pharmacological tools, as they are more informative and less error prone than single high concentrations. Most importantly, the limitations of existing inhibitors need to be carefully considered in the interpretation of experimental data. For example, the observation “the pathway X inhibitor Y in a concentration Z inhibited the response” is much more likely to stand the test of time than the bolder conclusion “pathway Y mediated the response.” Finally, it should be considered that a newly reported inhibitor often has been tested against a limited number of targets only, whereas inhibitors that have been around for a long time typically have been tested in a broader way. A cynical view of this would recommend using newly reported inhibitors early when they are still considered selective. A more scientific approach is careful interpretation of all inhibitor data.

While the above examples of promiscuous effects of pharmacological inhibitors are sobering and can perhaps even be concerning, they are not intended to discourage use of such compounds, as in many other cases they have proven valuable tools in the dissection of pathways mediating cell and tissue responses, for instance the role of PLC in muscarinic receptor agonist-induced contraction of the urinary bladder (65). Rather, investigators are encouraged to apply due diligence in choosing pathway inhibitors based on existing literature and not to blindly trust vague promises of companies selling fine chemicals. Moreover, a healthy degree of skepticism about unexpected findings is always a good approach; use of two or more inhibitors coming from distinct chemical families makes it less likely that effects are mediated by engagement of an unrelated target.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s). M. C. Michel currently is an employee of Boehringer Ingelheim. However, the present article has no relationship to this company or any of its products.

**AUTHOR CONTRIBUTIONS**

Author contributions: M.C.M. and R.S. conception and design of research; M.C.M. and R.S. analyzed data; M.C.M. and R.S. interpreted results of experiments; M.C.M. prepared figures; M.C.M. drafted manuscript; M.C.M. and R.S. edited and revised manuscript; M.C.M. and R.S. approved final version of manuscript.

**REFERENCES**

18. Böhmer T, Pfeiffer N, Gericke A. Three commercial antibodies against α1a-adrenergic receptor subtypes lack specificity in paraffin-embedded


Beta-arrestin activity 10.220.33.3 on October 4, 2017 http://ajpcell.physiology.org/ Downloaded from
Themes

C520 SELECTIVITY OF PHARMACOLOGICAL TOOLS


