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Mechanistic pharmacology, new developments

An industrial perspective on positive allosteric modulation as a means to discover safe and selective drugs

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Positive allosteric modulation is an innovative strategy for the discovery of drugs acting at 7-transmembrane receptors. Screening has led to the identification of numerous starting points for medicinal chemistry typified by novel mechanisms of action. The progression of compounds through hit-to-candidate phases and preclinical animal models, however, proves very challenging. In this review, we discuss advances in the area and interrogate the mechanistic profiling required to support drug discovery programs and fully exploit the therapeutic potential of positive allosteric modulators.

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Introduction

Positive allosteric modulation is of significant therapeutic interest for 7-transmembrane receptors (7TM) drug discovery. It is defined as the ability of a compound to potentiate the effect of an endogenous cognate ligand or other probes interacting orthosterically by binding at a distinct, 'allosteric', receptor recognition site. The compounds affect the physiological and/or pathological tone present *in vivo* at relevant sites of action (tissues/organs) through amplification of the endogenous agonist response. The modulation inherits spatial and temporal control of the biological response corresponding to the presence/absence of the endogenous ligand. The effects of the amplification are also saturable and potentially more selective, yielding the promise of safer therapies obtained by receptor activation. Positive modulation is a well-established phenomenon in the ion channel area and underlies the mechanism of action for classical drugs such as benzodiazepines. By contrast, the pursuit of positive modulation for 7TMs is less advanced but is gaining momentum following the approval of Cinacalcet (Sensipar™/Mimpara™) by the FDA in 2004 for secondary hyperparathyroidism and hypercalcaemia [1,2]. This article focuses on the promises and challenges associated with the progression of 7TM PAMs through the hit-to-candidate phases of drug discovery.

A novel paradigm for small molecule identification

During the early 00s, the 'screening all against all' (possible targets versus possible compounds) paradigm of discovery research was very much *in vogue*, boosted by breakthroughs from the human genome project. A move towards smaller target portfolios has since been observed across the pharmaceutical industry in an attempt to improve focus, reduce timelines and tackle drug attrition. 7TMs occupy a privileged position amongst molecular target classes with up to a 50% market share of current prescribed medicines [3]. They arguably represent the most important class of targets for the discovery of drugs and will probably continue to represent a significant proportion of target portfolios, particularly as

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methods improve for their prosecution. However, issues of chemical tractability have been encountered as the industry opened up beyond mainstay sub-families of 7TMs such as the serotonin (e.g. Sumatriptan/Imigran™, Fluoxetine/Prozac™), adrenergic (e.g. Carvedilol/Coreg™, Albuterol/Ventolin™, Salmeterol/Advair™), angiotensin (e.g. Losartan/Cozaar™), histamine (e.g. Cimetidine/Tagamet™, Ranitidine/Zantac™) and opioid (Hydrocodone/Vicodin™) receptors. Although some progress has been made, for example through the comparatively recent discovery of Maraviroc/Selzentry™, a CCR5 chemokine receptor antagonist, there remain many 7TMs with excellent link to disease but where the discovery of high-quality small molecules remains extremely challenging. In this respect, the adoption of functional assay readouts for primary screening has been truly enabling, leading to the identification of compounds with distinct underlying mechanism of action (MoA). The discovery of allosteric ligands has been on the rise over the past decade as exemplified by literature disclosures and patent filings for Family C 7TMs (Fig. 1). Whilst particularly true of Family C, the trend also applies to other families of 7TMs and therein supports the view of an overall improved chemical tractability. Examples of putative allosteric molecules have been extensively reviewed elsewhere [4–7], and some encouragingly show efficacy in animal models [8–11]. Allosteric molecules can be classified into negative (NAM), positive (PAM) or neutral modulators of the orthosteric agonist functional response(s) [12–17]. Allosteric molecules can also stimulate receptor activity directly and are so-called 'allosteric agonists', although instances of the latter are more rarely documented. Mixed profiles are referred to as 'ago-allosteric modulators'.

Positive allosteric modulation offers a new angle into drug discovery by further exploring the rich pharmacological potential of 7TMs (Table 1). The benefits of positive modulation are tangible in the processes of hit identification and follow-up medicinal chemistry. A growing body of evidence suggests a considerably higher probability of success identifying PAMs for targets with otherwise poor tractability when screened in agonist format. Given the same compound library is typically screened in both agonist and positive modulator mode, this suggests that the latter is inherently more attainable, presumably due to a greater complementarity between PAM binding sites and drug-like molecular structures. PAMs are typically quite drug-like and can diverge significantly in structure from the endogenous agonists, as would be anticipated for distinct binding modes. They probably encompass a diversity of putative MoA profiles. The rich mechanistic texture, when exploited, has the potential to provide much more subtle tools with which to probe the underlying biological hypothesis and guide programs towards 'desired' activity profiles for chemical optimisation [18]. Pure PAM molecules (devoid of agonism) bring an inherent specificity by means of the probe dependence (presence of orthosteric agonist) in the observed modulation response. Positive modulator screening is relatively simple and straightforward to prosecute [5,19,20]. It necessitates the use of appropriate assays sensitive to modulation. PAM tool molecules, when available, are extremely useful to guide

Table 1. Summary of cost–benefit analysis for pursuing PAMs during early drug discovery

Hit identification phase	Follow-up chemistry and biological validation in pre-clinical animal models
Benefits	
Approach is generic to all liganded receptors of the 7TM family	Ability to modulate endogenous tone at relevant tissues (spatial and temporal resolution)
More/potentially more drug-like chemical space available to exploit	Saturability of modulation in pharmacodynamic (PD) and behavioural animal models
Selectivity gain with respect to closely related sub-types	Potential to circumvent side-effects linked to agonism in a studies; safer biological profile, reduced likelihood of tolerance
Likelihood of diversity in putative MoA profiles, that is, potentiation of affinity and/or efficacy	Rich mechanistic texture can drive refinements in biological activity profiles, for example, functional selectivity, control of affinity and/or efficacy
Drawbacks	
Unprecedented approach in many cases; absence of tool molecules which can provide screening QC on sensitivity of modulation	Full appreciation of basal tone for endogenous ligand in target tissue/disease state is difficult
Requires use of native ligand(s) due to probe-dependency, which can make assay development more complex or costly	Correlation of activity between recombinant and native biology is often challenging
Orthologue activity may be lacking due to poor conservation of allosteric binding site; similarly, poor at predicting key liabilities	Relevant native assays are often hard to configure, that is, system-dependence, functional selectivity
Addressing response specificity is likely to entail multiple assays	Additional mechanistic complexity (*in vitro*, *in vivo*) can hinder program progression or delay it, for example, driving optimisation with multiple pharmacological parameters

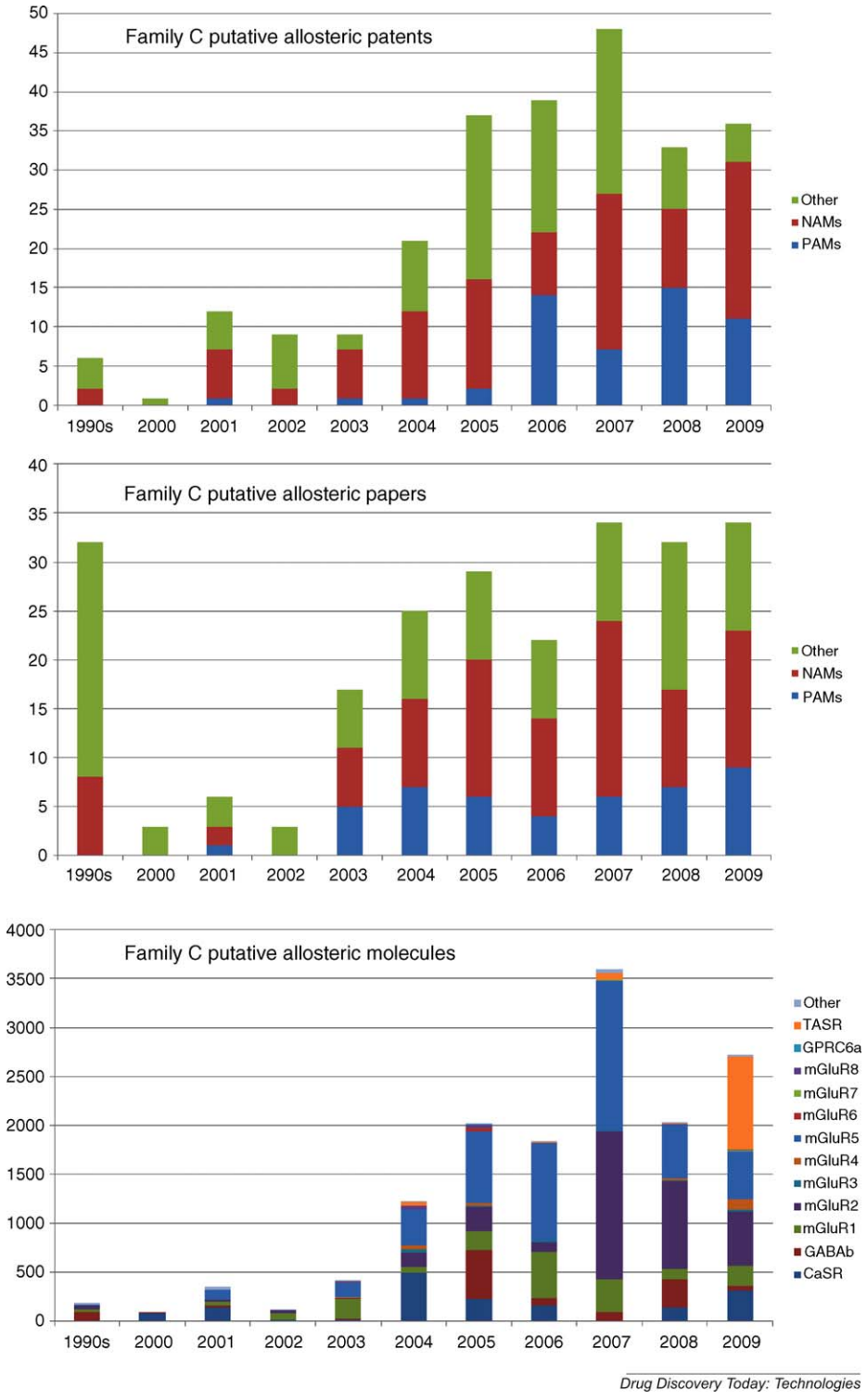

Figure 1. Explosion of reports for Family C 7TM allosteric ligands in the public domain demonstrating improved chemical tractability when screening using functional assays (NAMs) and screening in the presence of low concentration of orthosteric agonists (PAMs). Allosteric accounts within patents and papers which do not distinguish between negative and positive modulation are regrouped into 'other' (top and middle graph). Allosteric molecules reported without details on receptor subtype (e.g. mGluR) are regrouped into 'other' (bottom graph).

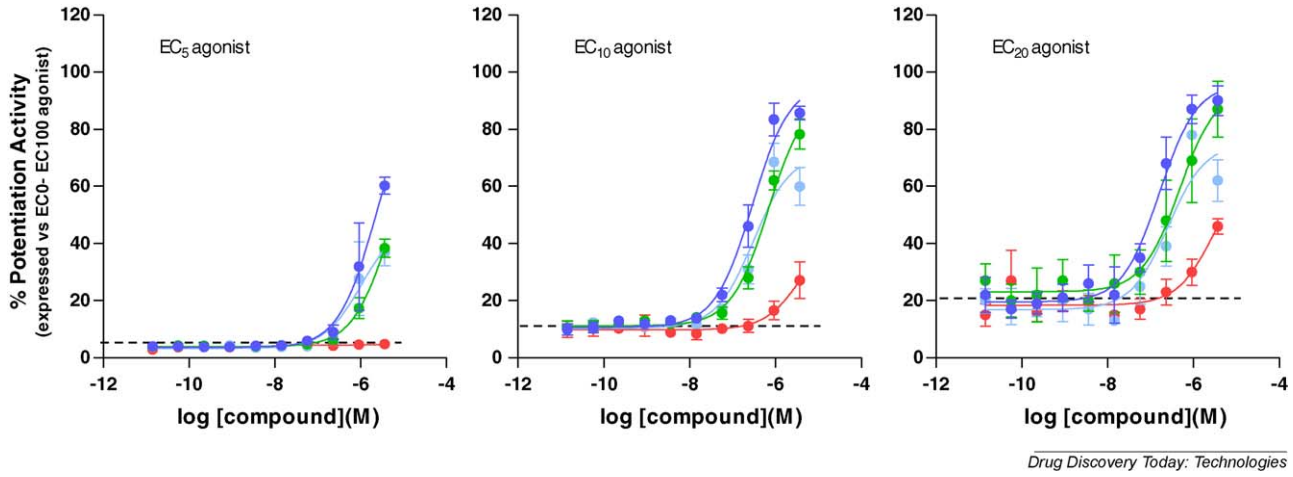

Figure 2. During screening, PAMs are typically tested as a function of a fixed agonist concentration corresponding to EC_{20}. The variability inherent to the measure of pEC_{50} of modulation in production screening can often be under-estimated. The impact associated with using lower than anticipated agonist concentrations over plate runs and/or days on assay sensitivity is significant. The graphs below illustrate the differential modulatory profiles obtained for four compounds derived from the same chemotype series when tested at agonist EC_5, EC_{10} and EC_{20}. Both potency and efficacy values appear to be affected to a different extent for each compound. In particular, it can be noted that one of the compounds is inactive at the lowest condition of agonist.

assay development and reagent validation. Assays are configured in the presence of low levels of the orthosteric agonist, typically set at EC_{20}, of the endogenous agonist rather than a surrogate whenever possible. The selection of agonist concentration is a balancing act between assay robustness and sensitivity of modulation (Fig. 2). Conventional assay readouts can be used; the ability to do so without the introduction of novel technology or platform results in comparatively simple logistics for implementation.

PAM mechanistic profiling

Bespoke assay solutions

The deconvolution of PAM activity for promising hits and the mechanistic support of follow-up chemistry call for significant screening effort. PAMs encompass a rich landscape of putative MoAs which can be broadly grouped based on their ability to promote affinity and/or efficacy of the agonist response. Key aspects under consideration for compound progression are summarised in Fig. 3. At first glance, it would appear that mechanistic profiling involves the generation of an alarming quantity of information and corresponding number of assays to be run. It follows that it is imperative to tailor mechanistic needs for programs on an individual-case basis, offer bespoke assay solutions and have biologists/chemists working hand-in-hand. Devising pragmatic ways in which to exploit such information is challenging but promises to advance medicinal chemistry and ultimately improve clinical efficacy and safety. Specificity of modulation can be demonstrated by testing PAMs in the presence of low concentrations of an agonist corresponding to an unrelated

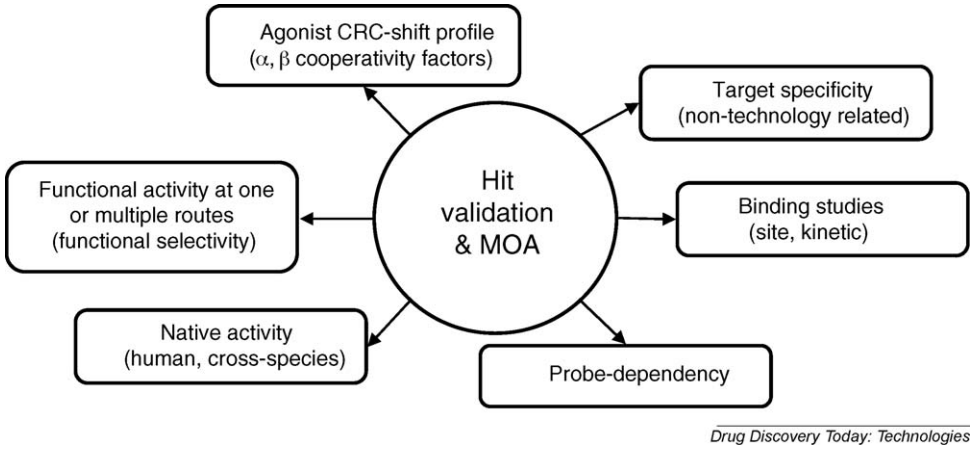

Figure 3. Outline of validation and mechanistic studies recommended for PAM molecules.

receptor endogenously expressed in the same cell background. Alternatively, or in combination, the 'orthosteric antagonist challenge' is an elegant approach which involves the selective blockade of modulatory response induced by PAMs via the dependency on the probe, that is, competitive steric hindrance of the probe at the orthosteric binding site (receptor subtype-selective antagonist). Binding assays are not routinely used during screening as allosteric radioligands are rarely available. Such a strategy is also limiting as it targets a specific allosteric site when others may exist. It can be argued that the mapping of the PAM binding site is not crucial to progress PAM molecules; but when possible, such information is valuable to support structure-based drug design (SBDD). Ligand kinetic studies can be enlightening regarding the duration of action of PAMs (on/off binding rates) and interplay with the kinetics of the orthosteric agonist, and is often an unappreciated aspect of significance.

Exquisite selectivity is achievable with PAMs over closely related receptor sub-types due to the exploitation of an allosteric site [21]. Such a site need not be conserved through evolution, although in turn need not be conserved between orthologues, leading to potential species issues in lead optimisation. Cases of disconnect between human, rat, mouse or other animal species have been encountered, highlighting the importance of testing for cross-activity before progressing compounds into animal models (Fig. 3) [22]. As opposed to setting up numerous orthologue assays ahead of understanding if there is orthologue disconnect, opting for a 'lite' screening approach may have great impact here and offer significant resource savings. This can be achieved by testing a handful of key exemplars within a chemotype at the onset of chemistry campaigns with transient assay systems. Issues identified can be followed up more fully and when appropriate.

Probe-dependency describes the relationship of the orthosteric agonist (or the so-called probe) and PAM molecule in driving cooperativity. This aspect is particularly relevant when multiple agonists are known for a given target, whether endogenous or surrogate small molecules. The phenomenon has profound implications on compound identification and optimisation. Hence, PAMs may selectively modulate surrogate orthosteric agonists but not the endogenous agonists. In this scenario, screening in PAM modality using a surrogate agonist may be misleading and irrelevant to *in vivo* modulation. On the contrary, probe-dependency can be advantageous in the case of multiple endogenous agonists or signalling pathways and provide a molecular framework for the modulation of selective biological systems *in vivo*.

Classification of MoAs

For many years, considerable effort has been made to refine theoretical models of drug–receptor interactions which can be employed to assess putative MoA profiles of PAM molecules [15,23]. The hallmark of PAMs is their ability to shift agonist concentration–responses to the left and/or up (affinity and/or efficacy) in a saturable manner. The MoA assay configuration therefore differs from the standard screening format where the agonist is typically fixed to a particular concentration. Both parameters of affinity (α) and efficacy (referred as β or ξ) of modulation can be quantified, although it should be noted that data interpretation can often be difficult and the power of the analysis limited by the accommodation of experimental data to theoretical curve fitting models. MoA studies are highly informative when comparing exemplars of chemical series or closely related compounds from the same series. However, the usefulness of this type of analysis for decision-making during structure–activity relationship (SAR) optimisation needs to be put into perspective. Benchmarking compounds in MoA studies is far more powerful when paired with validation studies in animal models. The pharmacological responses observed are dependent not only on compounds but also on assay systems used. This brings about the possibility of differing MoA profiles when comparing assay systems, that is, recombinant versus native settings. An example of this is agonism observed for some PAMs in recombinant assay systems where the receptor of interest is overexpressed. The presence of agonism may lead to a total masking of the positive modulation or an observed desensitisation effect. It can be hypothesised that agonism is directly linked to receptor expression levels, G-protein coupling or other aspects relating to the reconstituted cellular system used which do not necessarily mimic that of the *in vivo* situation. In cases of high receptor reserve where the agonist response reaches assay system maximum, compounds may drive apparent potency increases as a consequence of a modulation of efficacy rather than affinity. The ability to probe for functional responses in assay systems where receptor levels can be titrated may be particularly helpful when exploring mechanistic behaviours, that is, inducible stable cell lines or the BacMam delivery technology [24]. The integration of native/phenotypic assays to screening cascades when possible during SAR optimisation is also very informative.

Pluridimensionality and functional selectivity

Quantifying the effects of drugs is crucial to guide medicinal chemistry. Recent concepts of drug efficacy integrate advanced notions of pluridimensionality and functional selectivity driven by ligand–receptor binding [25–27]. In simple terms, it is possible for two ligands to elicit distinct responses whilst binding to the same receptor. Responses are complex and defined by the cumulative embrace of all signalling mechanisms, whether G-protein dependent or independent. As a result, small molecule ligands have the potential to induce effects that are not or only partially matched by the corresponding native agonist. PAMs have classically been defined by the ability to modulate affinity and/or efficacy of orthosteric agonists. This account is prob-

ably incomplete. PAMs may produce distinct receptor conformations and thereby potentiate the orthosteric agonist response, a subset of the response and/or drive other distinct functional events. Whilst this area remains to be clarified and is rather speculative, much more will no doubt be learned in years to come with the identification of further PAM small molecules. Functional selectivity may be valuable to target for the design of drugs. The promise of alleviating side-effects, for instance, at the same time as retaining the desired activity at the drug target is appealing [28]. However, this requires a grasp of the relative merit of signalling pathways in the recombinant assays typically in place to support SAR screening and their predictability of downstream endpoints. In practice, our understanding of the *in vivo* relevance of such routes is often limited. It is tempting to speculate that functional selectivity may in part explain *in vitro* versus *in vivo* pharmacology disconnects sometimes observed experimentally. It raises questions as to whether we currently appreciate the full spectra of efficacy of our compounds, be they PAMs or not, and how compound ranking should be performed during chemical optimisation.

Challenges with translational biology

Challenges with the progression of PAMs are in many ways shared with agonist molecules. Whilst agonists engage in classical paradigms of receptor activation, PAMs directly bind receptors to modulate such activation. Both act by selectively favouring a subset of receptor conformations amongst a landscape of possible behaviours, leading to cellular activity. Measured ligand efficacy (as opposed to the notion of intrinsic activity) is system-dependent, which makes the predictability of activity from recombinant to native settings far from straightforward. Where PAMs differ is in offering spatial and temporal control over activation in a saturable manner which brings the promise for safer drugs. The modulation of biological events at relevant tissues and time may confer reduced tolerance upon chronic drug exposure by avoiding issues typified of full activation, that is, desensitisation. Unfortunately, rarely are PAM tools available and extensively validated *in vivo* in relevant disease models at the start of drug discovery programs. Such information would be very valuable to guide the choice of mechanistic assays and screening strategies for progression of PAM molecules before animal models. The process is rather iterative, where compounds are first identified and then undergo rounds of optimisation in surrogate *in vitro* systems (i.e. recombinant or phenotypic) before *in vivo* testing; and vice versa (Fig. 4). There is almost certainly more biological complexity *in vivo* than is captured in *in vitro* screening assays. Nonetheless, the key is to integrate information to facilitate screening predictive of downstream endpoints. Detailed MoA studies in native assay systems are imperative to rationalise biological and chemical data from reconstituted or 'reduced' systems. Instances of direct receptor interactions with other proteins, whether they be with

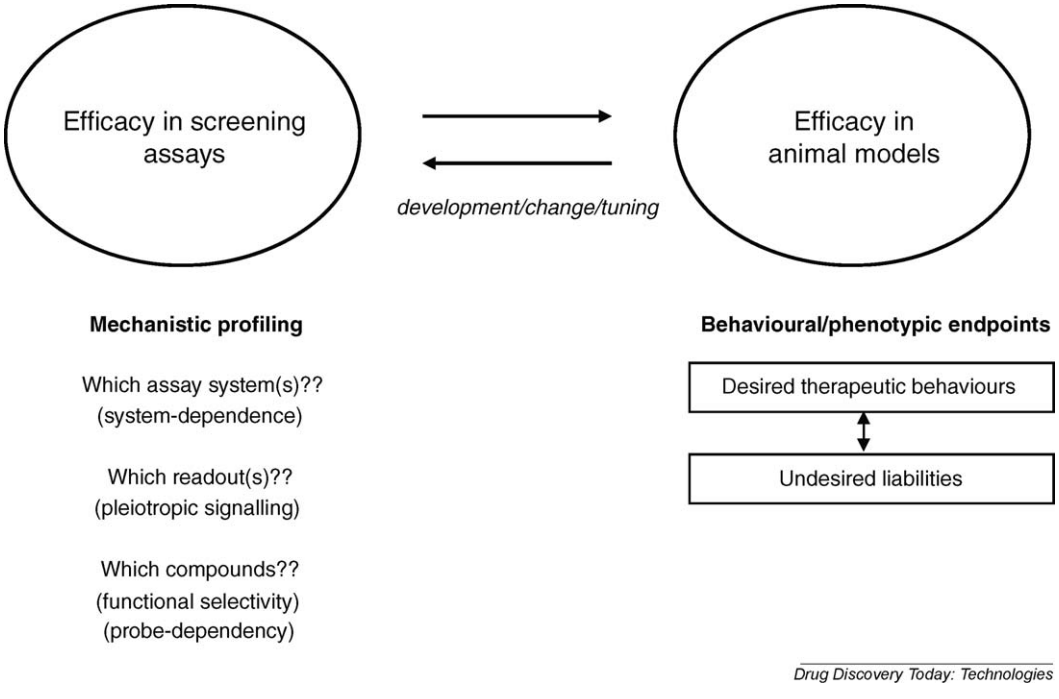

Figure 4. Support of medicinal chemistry efforts requires assays to measure PAM activity from *in vitro* to *in vivo* biological systems. The process of molecule optimisation is iterative and can last many years. Many questions underpin the choice of appropriate screening cascades, that is, assay type, readout, compounds.

membrane receptors (homo- and/or heteromerisation), intracellular signalling molecules (β-arrestin, G proteins, etc.) or something else, most probably contribute to compound efficacy in organs/tissues [29,30]. The choice of *in vivo* model(s) is difficult and cases of PAM molecules working in some but not all animal models may be encountered, reinforcing the subtleties within the biological systems under investigation and difficulty of data interpretation (especially lack of activity in animal models). Whenever possible, profiling in the most disease-relevant model(s) is preferred to better understand PAM activity profiles. It can ultimately lead to time and effort savings, especially given the low-throughput limitations entailed with animal work.

Endogenous tone at the site(s) of action is crucial for the effectiveness of PAMs *in vivo*. It is likely to differ in physiological versus pathological situations, potentially between species and even between individuals. This makes the validation of PAMs in animal models as well as predictions of efficacy across animal species (including humans) very challenging. A molecule tested in 'normal' animals under basal tonic conditions may not necessarily show any effect, illustrating the difficulty of tackling biological hypotheses with PAMs. Co-administration of agonist (native or surrogate) and PAM in normal animals may offer a paradigm by which to test the questions of probe-dependency and tone threshold. Moreover, the notion of tone present *in vivo* has significant ramifications and may also drive putative liabilities, off-target or otherwise, that were unforeseen. Spurious liabilities derived from cross-activation at unrelated targets may arise for some PAM molecules and probably be chemotype-specific. Screening for off-target activity on series of interest is typically achieved via a broad panel of recombinant assays. However, assays are rarely performed in allosteric mode and may have been performed historically with non-native probes, limiting the interpretation derived from cross-screening information. Target engagement is an important parameter for dose predictions in pre-clinical and clinical models. It relates receptor occupancy to effects at the relevant sites of drug action. In the case of positive modulation, the level of engagement needed is often poorly understood. The relationship between receptor occupancy and potency/efficacy of modulation is likely to be non-linear, at least at high PAM doses due to saturability of modulation or the so-called 'ceiling effect' driven by the cooperativity factor for the endogenous agonist. It is therefore tempting to assume that, in contrast to orthosteric agonist therapies, increasing receptor occupancy via a PAM will not lead to any detrimental effects. Interestingly, examples of clinically effective positive modulators acting at ion channels, that is, benzodiazepines have been described with relatively low potencies. A parallel can be drawn with the calcium-sensing receptor drug *Cinacalcet*, also associated with modest receptor potency (EC_{50} = 28–51 nM at 0.5 mM Ca^{2+} [31]). In this case, it should be said, however, that the tone (concentration) of the endogenous ligand is particularly high *in vivo*.

Concluding remarks

The search for 7TM PAMs as therapeutic agents is a relatively young field. So far, only a handful of cases for such molecules have successfully made it through lead optimisation and into the clinic (e.g. Cinacalcet/Sensipar™ marketed by Amgen and an mGluR2 PAM reported in Phase I development by Addex). Hence, it is difficult to draw conclusions about the rates of attrition in development for PAMs *versus* orthosteric molecules. In many instances, positive modulation also forms the basis of novel mechanistic hypotheses which do not have prior clinical validation in humans or pre-clinical evidence in disease animal models. Nonetheless, the prospect of PAMs to deliver activation of otherwise intractable targets as well as potentially safer and more selective drugs remains enticing. The discovery strategy appears to be generic to 7TMs by exploiting the natural propensity of the receptors to accommodate multiple binding sites. As such, it affords an opportunity to tackle previously intractable receptor sub-families within both Family A (e.g. SSTR2, NPY-Y2) and family B (e.g. GLP1, PTH1) as well as Family C receptors. Bespoke MoA support on key PAM compounds is essential and progression plans are likely to be complex. Classification of actives into clusters of MoA profiles paired with *in vivo* testing as early as possible during the lifetime of drug discovery programs will prove very powerful. Keeping an open mind will be important when examining MoA profiles as a compound with low potency and high efficacy of modulation may be desirable in certain circumstances whereas an opposite profile of high potency and low efficacy of modulation may be desirable in other situations. Probe-dependency and saturability of modulation will be paramount to the progression of PAMs and require early-on validation into biological systems. Targeted efficacy through positive modulation may offer potential for 'smarter' therapies with reduced liability and is, in particular, a promising prospect. To this end, alternative screening technologies such as label-free may be essential to complement the existing 7TM assay tool box and provide a phenotypic readout of cellular activity [32,33]. Lastly, when dealing with PAM molecules no assumption should be made as to 'what we don't know'.

Conflict of interest

The authors disclose no conflict of interest.

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