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Perspective

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2013 Philip S. Portoghese Medicinal Chemistry Lectureship: Drug Discovery Targeting Allosteric Sites†

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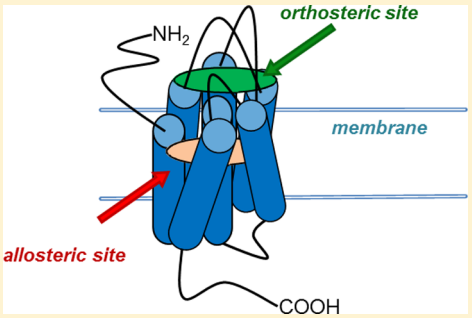
ABSTRACT: The identification of sites on receptors topographically distinct from the orthosteric sites, so-called allosteric sites, has heralded novel approaches and modes of pharmacology for target modulation. Over the past 20 years, our understanding of allosteric modulation has grown significantly, and numerous advantages, as well as caveats (e.g., flat structure−activity relationships, species differences, "molecular switches"), have been identified. For multiple receptors and proteins, numerous examples have been described where unprecedented levels of selectivity are achieved along with improved physiochemical properties. While not a panacea, these novel approaches represent exciting opportunities for tool compound development to probe the pharmacology and therapeutic potential of discrete molecular targets, as well as new medicines. In this Perspective, in commemoration of the 2013 Philip S. Portoghese Medicinal Chemistry Lectureship (Lindsley, C. W. Adventures in allosteric drug discovery. Presented at the 246th National Meeting of the American Chemical Society, Indianapolis, IN, September 10, 2013; The 2013 Portoghese Lectureship), several vignettes of drug discovery campaigns targeting novel allosteric mechanisms will be recounted, along with lessons learned and guidelines that have emerged for successful lead optimization.



I. INTRODUCTION: BACKGROUND ON ALLOSTERIC MODULATION. NOVEL APPROACHES FOR THERAPEUTICS

While the first concepts regarding allosterism were put forth in the 1960s, only in the past decade, with advances in molecular pharmacology and functional screening technology, has the impact of this alternative approach for target modulation been realized.[2−12] Indeed, the discovery of topologically distinct allosteric (from the Greek as "other site") binding sites for a diverse range of receptor and protein families (GPCRs, ion channels, caspases, kinases, and phospholipases) has provided unparalleled opportunities to obtain druggable small molecules with exquisite selectivity and unique pharmacological profiles.[2−12] Here, an allosteric ligand binds the target at a topographically distinct allosteric site and either potentiates or inhibits the binding and/or signaling of an orthosteric ligand by taking advantage of conformational flexibility of the receptor and/or protein.[2−12] The clinical success and safety of benzodiazepines (BZDs) **1**−**3** (Figure 1), the first allosteric modulator drugs, which potentiate the effect of γ-aminobutyric acid (GABA) at the ionotropic $GABA_A$ receptor are in direct opposition to the adverse and potentially lethal effects of orthosteric $GABA_A$ agonists.[4,11,13] Further exploration within the BZD class elucidated multiple modes of allosteric pharmacology: positive allosteric modulators (PAMs), which potentiate $GABA_A$ receptor response, negative allosteric modulators (NAMs), which decrease channel activity, and silent allosteric modulators (SAMs, or no affect ligands, NALs) that bind to the allosteric site and block both PAM and NAM activity without any effect on receptor signaling alone.[4,11,13] These data fueled the concept of allosteric modulation in modern drug discovery leading to the identification of allosteric modulators for other ion channels, kinases, phospholipases, and G-protein-coupled receptors (GPCRs).[11,13,14] Moreover, multiple allosteric modulators are now in various stages of clinical development[11,13,14] as well as marketed therapeutics (cinacalcet, **4**, a calcium sensing receptor PAM, and maraviroc, **5**, a CCR_5 NAM).[15,16]

Over the past 13 years, our laboratories at Merck and within the Vanderbilt Center for Neuroscience Drug Discovery (VCNDD)[17] have pioneered allosteric modulation as a pharmacological approach to modulate kinases, GPCRs, ion channels, and phospholipases,[11,13,14] and we have introduced a plethora of important small molecule tools for use by the biomedical research community (via the VCNDD and the Molecular Libraries Probe Center Network, or MLPCN).[17,18] Clearly, allosteric ligands afford unprecedented selectivity (by targeting evolutionary less conserved binding sites), enhanced chemical tractability, and improved physiochemical properties.[2−12] In the course of our research programs, we have encountered numerous caveats surrounding allosteric ligand pharmacology and chemical optimization (ligand bias, species differences, "molecular switches", flat SAR, the "fluorine walk") for which we have developed guidelines and strategies to enhance the odds of a successful lead optimization campaign.[2−12,14] These general concepts have all been

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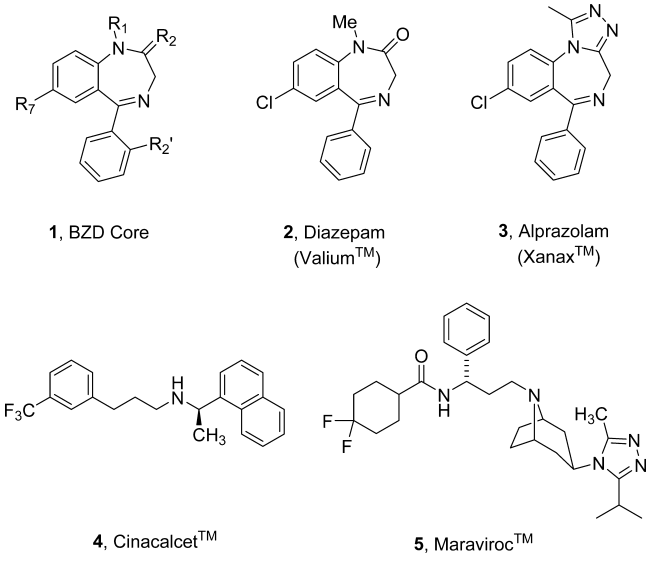

Figure 1. The first allosteric modulators with clinical success were benzodiazepines (BZDs), $GABA_A$ PAMs. The generic BZD core **1** and important medications **2** (Valium) and the tricylic analog **3** (Xanax) are shown. Also shown are structures of the two marketed GPCR allosteric modulators: cinacalcet (**4**), a calcium sensing receptor PAM, and maraviroc (**5**), a CCR_5 NAM.

extensively reviewed elsewhere;[11,12,14] thus, this Perspective will focus on the defining allosteric modulator programs that gave rise to these principles along with programs that transitioned from conceptual preclinical postulates into human clinical trials.

II. ALLOSTERIC MODULATION OF KINASES: THE CASE OF Akt

Protein phosphorylation is a ubiquitous cellular signaling process mediated by the action of kinases, and dysfunction within this system gives rise to numerous human diseases from diabetes to cancer.[19−21] Despite the presence of >500 kinases in humans and a highly conserved orthosteric ATP (**6**) binding site, numerous small molecule drugs have received FDA approval; however, selectivity versus the kinome remains a major challenge, especially for kinase family with multiple isoforms/isoenzymes.[22,23] Today, we recognize four major types of kinase inhibitors: type I (classical ATP mimetics), type II, (ATP-site binders that extend into an adjacent allosteric site), type III (bind exclusively at an allosteric site, near the ATP-site), and type IV (bind exclusively at an allosteric site, distinct and remote from the ATP-site).[24] Type IV inhibitors pharmacologically can induce structural reorganization, stabilize inactive conformations, prevent substrate recognition, induce degradation, and/or occupy autoinhibitory sites by targeting a limited number of inactive kinase conformations, such as disruption of a conserved αC-helix.[25−28] To date, allosteric kinase inhibitors have been developed for Akt, Abl, JNK1, mTOR, CDK2, CHK1, IGF-1R, and PDK1, which possess chemotypes distinct from classical ATP mimetics and excellent selectivity versus the kinome.[29] However, with respect to a nascent Akt kinase inhibitor program at Merck in early 2001, this was virgin territory and to pursue a novel, nontraditional (not ATP-competitive ligand) approach, was viewed with skepticism.

In 2001, the modulation of discrete signaling targets in PTEN/PI3K pathway was a major focus for oncology drug discovery.[30] At this time, Akt (PKB), a serine/threonine kinase in the AGC family of kinases (e.g., PKA, PKC, SGK), was known to be an oncogene, and an attractive kinase target though small molecule ligands were classical ATP-competitive, typified by the pan-kinase inhibitor staurosporine **7** or lipid analogs such as **8** (Figure 2).[30−32] In mammals, there are three isoforms/isoenyzmes of Akt (Ak1, Akt2, and Akt3) with different physiological roles that share greater than 85% homology (>95% at the ATP pocket). Structurally, each Akt isoform contains an N-terminal pleckstrin homology (PH) domain, a kinase domain, and a C-terminal regulatory domain. Akt was known to be comformationally flexible existing in the cytoplasm in a closed, inactive PH-in conformation, where the PH domain shields the ATP binding pocket and blocks phosphorylation of Ser473/T308.[30−37] Upon PIP3 recruitment to the plasma membrane, Akt then adopts a PH-out conformation that exposes Ser473/T308 for phosphorylation by PDK1 and mTORC2 (in 2001, it was believed to be a putative PDK2).[30−37] Could this unique conformational flexibility of Akt engender allosteric sites that could effectively "lock" Akt into an inactive (PH-in) conformation and thus inhibit both the activity and the activation of Akt (Figure 3A)? Beyond this critical question, the objectives for this nascent project involved the development of small molecule tools to assess the apoptotic response of selective inhibition of Akt1, Akt2, and dual Akt1/2 inhibition, and once clear, the development of an oral Akt clinical candidate for the treatment of cancer. Notwithstanding the issues surrounding kinome selectivity, also developing Akt inhibitors with isoform selective inhibition by targeting the ATP-binding site seemed improbable and unlikely, as no selective Akt kinase inhibitors were known, and no one had yet attempted to achieve isoform selective inhibition of Akt. Thus, we chose a different and unprecedented, for that time, approach.

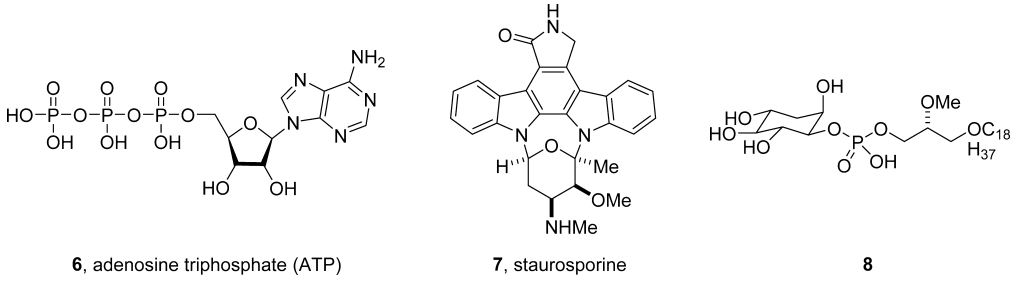

Figure 2. Structures of adenosine triphosphate (ATP) **6**, the prototypical ATP-competitive, pan-kinase inhibitor staurosporine **7**, and a PIP lipid analog **8**. In 2001, **6** and **7** were examples of known Akt inhibitors, but neither were selective versus the kinome or other PH-domain containing proteins.

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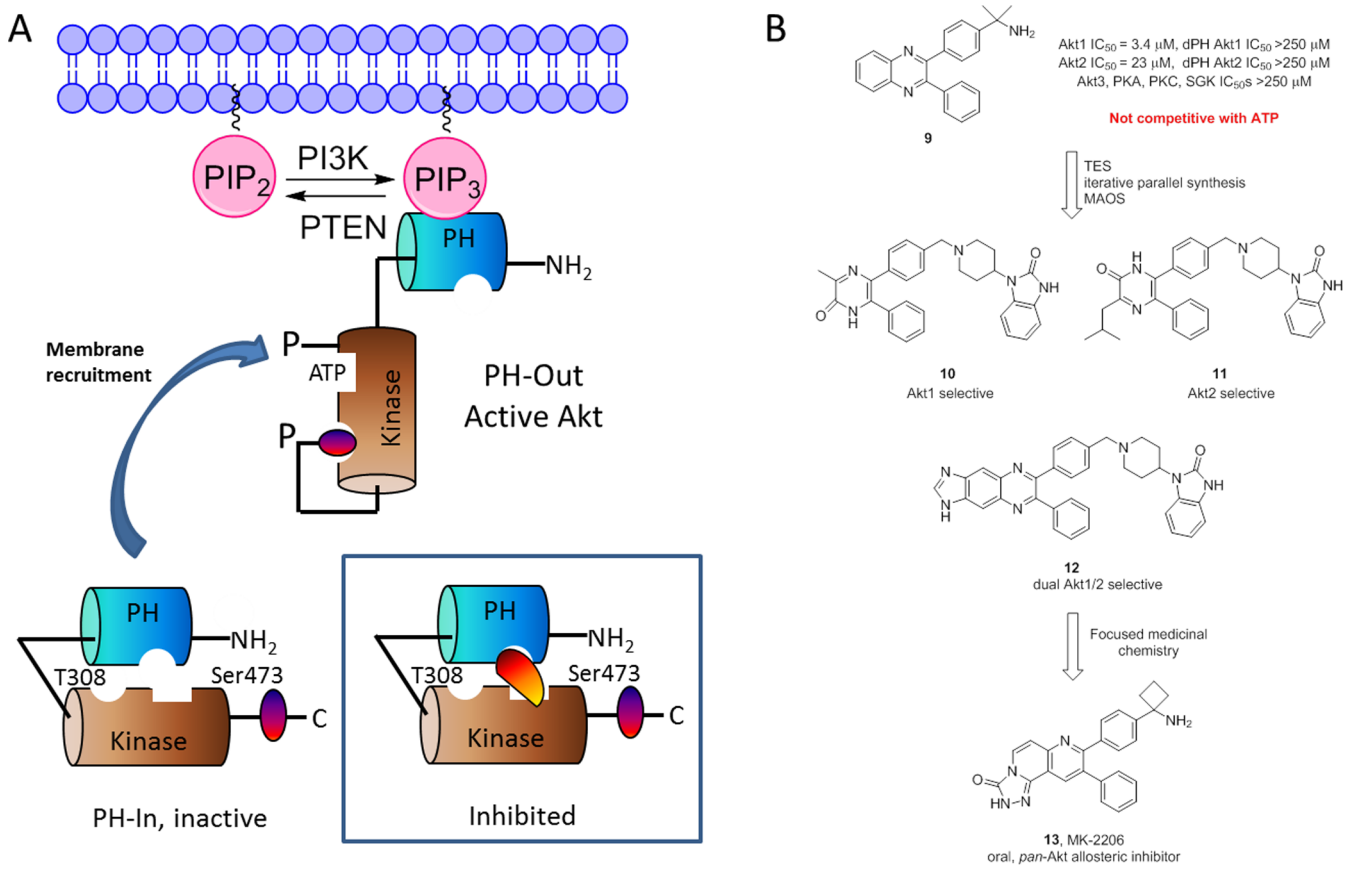

Figure 3. Akt biology and allosteric inhibitors. (A) Loss of function mutations or deletions in PTEN leads to high levels of PIP3 which recruits Akt from the cytoplasm, where it exists in an inactive, PH-in conformation to the plasma membrane, where the PH domains binds to PIP3 leading to a PH-out conformation exposing T308 and Ser473 for phosphorylation by PDK1 and mTORC2, and active Akt. Inset: a model by which an allosteric inhibitor could stabilize the PH-in conformation, thus blocking both the activity and activation of Akt. Detailed mutagenesis, biochemical, and later X-ray studies confirmed that allosteric Akt inhibitors **9**–**13** adhere to this model of inhibition. (B) HTS lead **9** was found to be the first allosteric inhibitor of Akt. An iterative parallel synthesis and MAOS approach (TES) delivered key tool compounds with unprecedented selectivity for Akt1 (**10**), Akt2 (**11**), or both Akt1 and Akt2 (**12**). Subsequent intense medicinal chemistry provided MK-2206 (**13**) an orally bioavailable pan-allosteric Akt inhibitor that advanced into clinical trials and displayed efficacy against solid tumors in phase II.

A high-throughput screen (HTS) was performed, and as expected, the "usual suspects" (ATP mimetics) resulted. An exceptional biochemist on the program, Stanley Barnett, then performed ATP kinetics with the HTS hits and found a lone compound **9**, a functionalized quinoxaline, that was non-competitive with ATP and was dissimilar from classical ATP-competitive ligands (Figure 3B).[38] Moreover, **9** was active at Akt1 and Akt2 but inactive (IC_{50} values of >250 μM) at Akt3 or against Akt 1 and Akt2 mutants lacking the PH domain as well as PKA, PKC, and SGK. Moreover, **9** inhibited both the activity and the activation of Akt. These data strongly suggested that the binding site for **9** resided within the PH domain or required the PH domain, thus suggesting an allosteric mode of inhibition.[38] Extensive mutagenesis work and biochemical studies supported a model wherein **9** was a two-site, allosteric binder, with a high affinity site in the PH domain, inducing a conformational change to a second site in the catalytic domain, locking Akt into a closed, PH-in conformation.[38] As this was a nascent program, chemistry support was limited to a single medicinal chemist, but significant optimization of **8** was required to meet the program objectives. Here, we developed the technology enabled synthesis (TES) approach of iterative parallel synthesis and fragment libraries, coupled with microwave-assisted organic synthesis and mass-directed preparative LCMS, to leverage technology for accelerating lead optimization with limited human resources.[39] In short order, we had identified a key piperidine benzimidazole moiety that significantly enhanced Akt activity along with cores that afforded the required Akt1-selective **10**, Akt2-selective **11**, and dual Akt1/2 selective inhibitors **12**.[36] Like **9**, **10**–**12** were PH-domain dependent inhibitors, noncompetitive with ATP, and allosteric. Early proof-of-concept studies demonstrated that inhibition of both Akt1 and Akt2 was required for maximal apoptotic effect and that **12** could inhibit both basal and IGF-stimulated Akt1 and Akt2 phosphorylation in mouse lung.[36] Effort then focused on development of a clinical candidate[32] and addition of a larger medicinal chemistry team that ultimately delivered MK-2206 (**13**), the first allosteric, oral Akt kinase inhibitor that enhanced antitumor efficacy by standard chemotherapeutics and was efficacious in patients with solid tumors in phase II (Figure 3B).[40] Years later, the proposed mechanism of Akt inhibition was confirmed both by X-ray crystallography, whereby a cocrystal of Akt and **12** illustrated that **12** binds in a hydrophobic pocket formed by residues within the PH and kinase domains, and by fluorescence resonance energy transfer (FRET) data showing that **12** locked Akt into a PH-in conformation, preventing phosphorylation of S473 and T308.[14,41] Serendipitously, the

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Chart 1

Box 1. Guiding Principles for Allosteric Modulator Optimization

- **Primary Assay.** 'Triple add' protocol for the primary assay (capture 'molecular switches')
- **Molecular Switches.** Avoid series that display a strong tendency for mode/subtype switching. If pursued, rigorously identify, re-synthesize and pharmacologically characterize the major metabolites as they may possess differing pharmacology/selectivity.
- **Lead Optimization Paradigm.** Iterative parallel synthesis, matrix libraries (e.g, 6 x 6), the 'flourine walk' and deuterium incorporation all work in an integrated fashion to overcome the shallow or flat SAR of allosteric ligands and improve DMPK.
- **Ago-PAM Activity.** Depending on the target, ago-PAM activity can be desired or must be avoided. Lead optimization campaigns should be driven using cell lines with low receptor reserve, and when possible, cross-check in native systems (e.g., astrocytes, E-phys).
- **Stimulus Bias.** Try to avoid series with strong stimulus bias, in favor of allosteric ligands that modulate all signaling, akin to the orthosteric ligand, unless data suggest stimulus bias is required. If this case, the assay should be high-throughput to support chemistry.
- **PET Ligands.** Develop PET ligands within the same chemical series as the candidate. Due to cooperativity issues, PET tracers more readily developed from NAMs and NALs (SAMs) than PAMs.
- **Selectivity Screening.** In addition to classical radioligand binding panels to assess ancillary pharmacology, key compounds should be profiled in functional GPCR assays with full agonist CRCs.
- **Species Differences.** Cell lines for your GPCR target are required across safety species (rat, dog, NHP) to ensure no species differences that could hinder GLP Tox studies.

allosteric approach with **13** also enabled our project team to side-step a major issue only recently disclosed. Upon binding, ATP-competitive Akt inhibitors induce hyperphosphorylation engendering regulatory phosphorylation; in contrast, **13** inhibits drug-induced hyperphosphorylation,[42] thus increasing the therapeutic window noted for **13** and newer back-up compounds from Merck. Overall, this first venture into allosteric drug space was fruitful and demonstrated the value of a commitment to basic science and novel approaches for target modulation to advance into the clinic and paved the way for a career focused on allosteric mechanisms of target modulation.

III. ALLOSTERIC MODULATION OF GPCRS

G-protein-coupled receptors, also referred to as seven-trans-membrane receptors (7TMRs), account for >50% of all known drugs, and with the exception of **4**[15] and **5**,[16] the remaining FDA-approved drugs bind at the orthosteric site and modulate receptor function by blocking the action of the native agonist (competitive antagonism), inhibition of constitutive activity (inverse agonism), or direct activation (agonism).[2−12,14] The historical reason for this trend lies in the assays employed in their discovery: radioligand displacement assays that targeted the orthosteric site.[2−12,14] Despite this success, many GPCRs are still without ligands and selectivity remains an issue, with desensitization resulting from prolonged activation, and based on the nature of the orthosteric ligands (neurotransmitters (family A), amino acids (family C), and large peptides (family B)), ligands often possess poor physiochemical properties (especially for CNS targets). With the advent of high throughout functional assays, it became possible to identify ligands that modulate GPCR function without regard to the binding site, which heralded the dramatic growth of allosteric modulators and a new frontier in pharmacology.[2−12,14] Like the BZDs,[13] GPCRs allosteric modulators can act as PAMs, NAMs, NALs (SAMs), ago-PAMs, partial antagonists, and even allosteric agonists (activating the GPCR in the absence of native agonist). It has been demonstrated time and again the advantages of allosteric modulation: (1) both subtype and overall selectivity, (2) maintenance of activity dependence (state dependence), (3) temporal and spatial aspects of endogenous physiological signaling, (4) less densensitization, and (5) fewer side effects. However, there are also challenges: (1) steep shallow SAR, (2) species differences (due to less evolutionary conservation of allosteric sites), (3) signal bias, (4) "molecular switches", (5) allosteric ligands that can act at multiple distinct, overlapping, and nonoverlapping sites on the same receptor, and (6) the impact of homo- versus heterodimer pharmacology.[2−12,14] This field has been extensively reviewed in a *Journal of Medicinal Chemistry* Perspective[11] as well as in multiple other venues;[2−10,12,14] therefore, this section will serve to highlight the GPCR allosteric modulator programs from our laboratories that helped establish this field and defined guiding principles (Chart 1) for allosteric ligand optimization.

Once again, we return to Merck in 2001, where Jeff Conn was the Director of Neuroscience and my medicinal chemistry group was supporting his nascent programs targeting metabotropic glutamate receptors (mGlus) where the orthosteric agonist is glutamate (**14**).[43] At this time, $mGlu_5$ NAMs, such as MPEP (**15**), were well-known (Figure 4),[44] and Dr.

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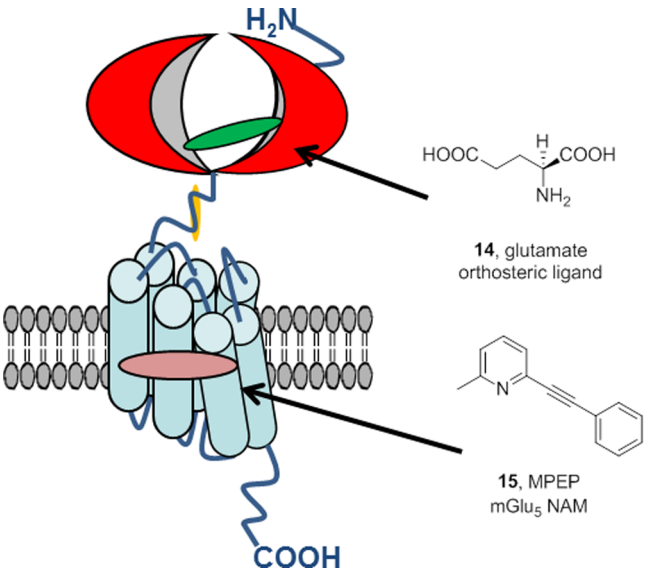

Figure 4. Cartoon of the metabotropic glutamate receptor subtype 5 ($mGlu_5$) showing the structure and binding site of the endogenous agonist glutamate (**14**) in the extracellular Venus fly trap domain and the prototypical allosteric ligand, MPEP (**15**) an $mGlu_5$ NAM, that binds within the transmembrane domain.

Conn postulated that $mGlu_5$ PAMs should then be pharmacologically possible. If so, PAMs could avoid the epileptiform activity of $mGlu_5$ agonists[43] and represent a potential novel therapeutic mechanism, via the NMDA hypofunction hypothesis,[45] to improve cognition and treat schizophrenia.

However, there were no reports of $mGlu_5$ PAMs, and management was not supportive of an HTS campaign. Therefore, to initiate a PAM effort, we examined the historical $mGlu_5$ NAM HTS data and looked for compounds that potentiated the glutamate EC_{80} (as opposed to the classical PAM EC_{20} screening paradigm). Despite "noisy" data, we identified two compounds worthy of follow-up. The first was a benzaldazine series represented by DFB (**16**), the first $mGlu_5$ PAM (EC_{50} = 2.6 μM), with the anticipated PAM pharmacology and was shown to bind at the well-characterized MPEP NAM site (Figure 5).[46] SAR around **16** was surprising in that subtle changes led to an equipotent mGlu5 NAM (**17**) and **18**, the first $mGlu_5$ SAM (now termed a NAL).[46] At this point, we had yet to see this as a general phenomenon which we later (in 2008) coined "molecular switches",[47−49] but rather it was viewed as an anomaly. Interestingly, the other hit **19** also behaved as a PAM and potentiated NMDA receptor currents, but pharmacological characterization indicated that it, as well as an advanced $mGlu_5$ PAM in this series CPPHA (**20**),[50,51] did not bind at the MPEP site, thus providing evidence that there are two allosteric sites on $mGlu_5$ that can enable positive allosteric modulation of the receptor. In parallel to the iterative parallel synthesis that delivered **20**, we also performed fragment libraries around **20** that led to CDPPB (**21**), the first centrally active $mGlu_5$ PAM that validated this novel mechanism in preclinical models of schizophrenia and cognition.[52,53] Surprisingly, **21** was shown to bind at the MPEP site, and many other laboratories utilized **21** to demonstrate antipsychotic-like effects in multiple animal models as well as enhancement of synaptic plasticity and cognitive function. However, Merck elected to stop the $mGlu_5$ PAM program in favor of other schizophrenia programs.

After these initial reports, multiple companies and academic laboratories initiated $mGlu_5$ PAM programs, and now there are over 40 distinct chemotypes reported as $mGlu_5$ PAMs, both for the MPEP and for the non-MPEP binding sites, with increasing examples of therapeutic efficacy.[54] Upon moving to Vanderbilt to once again partner with Jeff Conn and establish the VCNDD,[17] we initiated a new $mGlu_5$ PAM project with a full "triple add" HTS campaign,[2−12,14] which afforded a plethora of fundamentally new $mGlu_5$ PAM and NAM leads. Key to our new Center was to have a dedicated, NIH-funded basic science effort to parallel the drug discovery effort that generated in vivo tool compounds that enabled a "deep dive" into target biology to inform the drug discovery teams. Shortly after developing patented $mGlu_5$ PAMs, we partnered the program with Janssen and quickly developed a broad intellectual property suite en route to a new medical entity (NME), while our basic science team provided keen insights into the biology of $mGlu_5$ potentiation. For example, we identified $mGlu_5$ PAMs that closely resembled MPEP, yet did not bind in a competitive manner with the MPEP binding site, leading us to realize that PET tracers for the program had to be generated from within the exact same scaffold as the candidate to ensure translational utility (Chart 1).[54] Furthermore, a critical observation with MPEP site ligands, as an extension to the early observations with **16**−**18**,[46] was a general phenomenon we coined "molecular switches".[47−49] Here, we noted that the mode of pharmacology of an $mGlu_5$ partial antagonist **22** could be switched to a PAM **23** by the addition of a methyl group in the 4-position of the distal phenyl ring (Figure 6). The addition of a 2-aminomethyl group to the 2-position of the pyrimidine provided **24**, a potent $mGlu_5$ PAM that displayed in vivo efficacy in antipsychotic models.[48] All other structural modifications to **22** engendered full $mGlu_5$ NAM activity. Other series proved impervious to "molecular switches", and these series were more favorable as leads toward an NME. As we will discuss later, the phenomenon of "molecular switches" is not only limited to $mGlu_5$ or class C GPCRs but is also prevalent with allosteric ligands for class A and class B GPCRs as well.[12,14,49]

The observation that incorporation of a small, polar moiety could engender a "switch" in the mode of pharmacology caused serious concern regarding the potential for CYP-mediated "molecular switches" from oxidative metabolism. Here, the academic effort with "tool compounds" informed the drug discovery team to judiciously characterize metabolites. About this time, Merck reinitiated an $mGlu_5$ PAM program through a partnership with Addex Pharmaceuticals and had published a report on mechanism-based toxicity based on data within a series of $mGlu_5$ PAMs, represented by 5PAM523 (**25**).[55] In this study, fluorojade staining showed necrotic neurons in the auditory cortex and hippocampus (Figure 7); moreover, these findings, in part, led Merck to once again abandon their $mGlu_5$ PAM program. We were aware of this, as well as the potential for neurotoxicity and seizure liabilities (known for group I mGlu agonists) with PAMs that possessed agonist activity, e.g., ago-PAMs. Once again, our academic, deep science effort realized the need for both high- and low-expressing $mGlu_5$ cell lines as well as the need to have a native preparation, which was found in astrocytes (they natively express $mGlu_5$). For PAMs such as VU0242465 (**26**) which displayed ago-PAM activity in both cell lines and also displayed $mGlu_5$ agonism in astrocytes,

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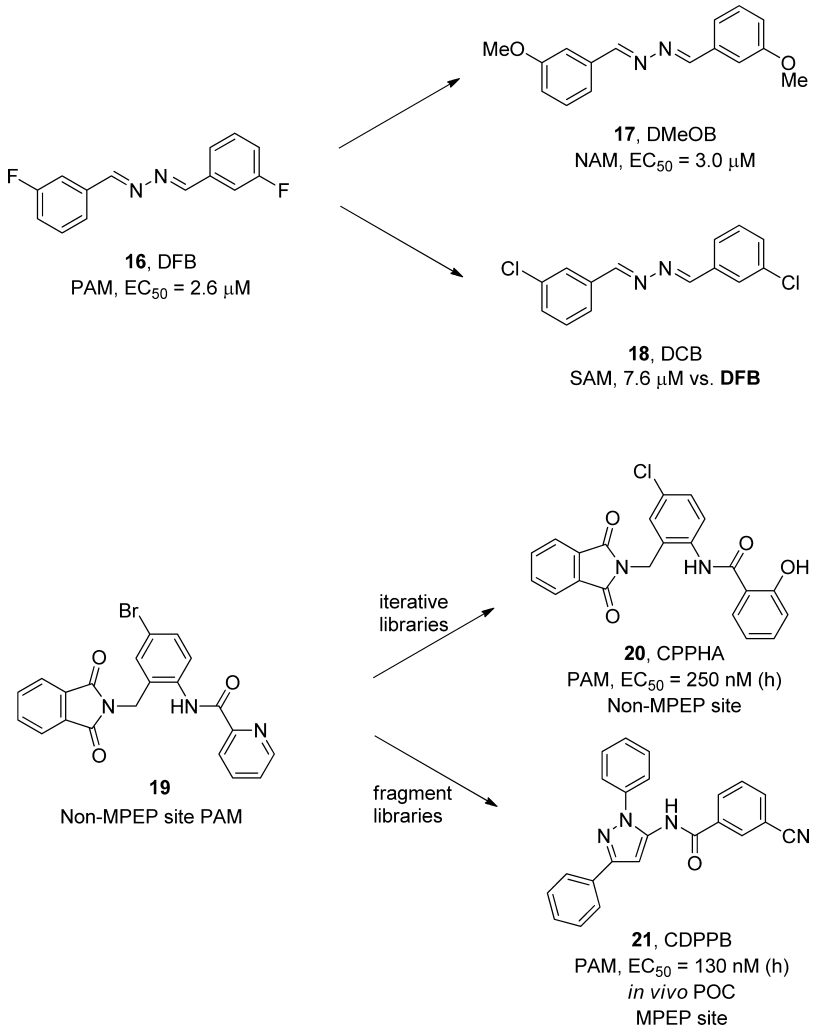

Figure 5. The first $mGlu_5$ PAMs **16** and **20** and the subsequent optimization that led to the discovery of two distinct allosteric sites on $mGlu_5$ that can potentiate receptor function and the key in vivo proof of concept $mGlu_5$ PAM **21** that validated the mechanism and pharmacological approach for treatment of multiple symptom clusters of schizophrenia.

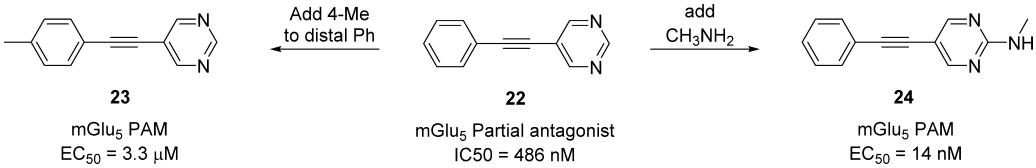

Figure 6. "Molecular switches" within MPEP site $mGlu_5$ allosteric ligands. Subtle structural modifications modulate the mode of pharmacology. **24** proved to be a potent PAM with in vivo activity in antipsychotic models, demonstrating the mode of pharmacology "switch" in vitro was mirrored in vivo.

we knew they potentiated long-term depression (LTD) alone and induced behavioral disturbances (seizures) when dosed in rats and were to be avoided. However, we were very surprised when VU0403602 (**27**), a pure PAM both in cell lines and in astrocytes, was found to induce significant seizure activity.[56] Studies with MTEP demonstrated that the adverse seizure activity was $mGlu_5$ mediated, with a full blockade of seizure activity with 10 mg/kg MTEP. On the basis of our concern of CYP-mediated "molecular switches" and the presence of a hydroxyl moiety in the amide of **26**, we also pretreated rats with aminobenzotriazole (ABT), a pan-CYP inhibitor, prior to **27** and found that the seizure activity was completely suppressed and equivalent to 10 mg/kg MTEP. Thus, the seizures were $mGlu_5$ mediated, most likely $mGlu_5$ allosteric agonism, and the activity was due to a metabolite and not the parent **27**. Metabolic identification studies then showed that the major metabolite M1 (**28**) was due to oxidation of the cyclobutyl moiety, and this secondary alcohol proved to be a potent $mGlu_5$ allosteric agonist, a similar moiety to that found in **26**. NOE studies and synthesis later demonstrated that there was also stereochemical bias in the "molecular switch" with the trans-isomer **29** possessing potent (EC_{50} = 400 nM) allosteric agonist activity, while the cis-isomer **30** remained a pure PAM (EC_{50} = 33 nM).[56] Thus, this was the first reported example of an in vivo, CYP-mediated "molecular switch", and subsequent

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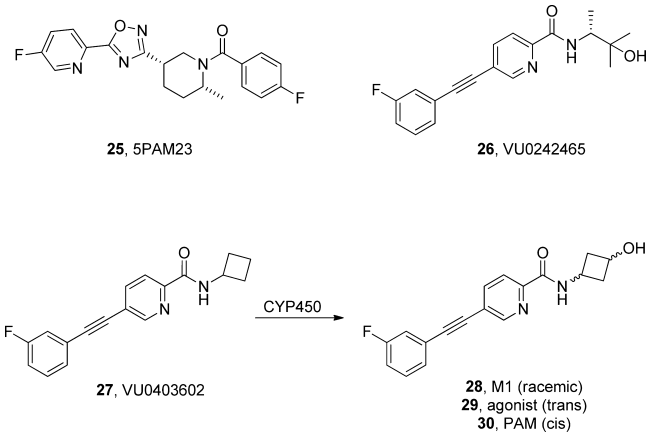

Figure 7. $mGlu_5$ PAMs and ago-PAMs leading to seizure liability and neurotoxicity. Pure PAM **27** also induced seizures, but studies found that a CYP-mediated "molecular switch" afforded a racemic secondary alcohol metabolite **28** that modulated the mode of pharmacology from pure PAM to allosteric agonist and gave rise to the adverse effect liability and not the parent.

allosteric modulator programs now closely monitor major metabolites and their pharmacology/selectivity.[56]

Finally, the concept of stimulus/signal bias was well-known in the context of GPCR allosteric modulators, and in the case of $mGlu_5$, potentiation of NMDA receptor currents represented the major adverse effect liability once allosteric agonism had been addressed. Under this principle, we evaluated several $mGlu_5$ PAMs and found a compound that did not potentiate NMDA receptor currents directly, yet displayed robust efficacy, akin to $mGlu_5$ PAMs that potentiated NMDA receptor currents, in preclinical rodent models of antipsychotic activity, potentiated hippocampal LTD and LTP and yet showed no acute adverse effects or short-term excito- or neurotoxicity (fluorojade staining) in rats, in contrast to **25**;[1] however, these results do not ensure an absence of toxicity in other species or upon higher and/or chronic dosing regimens.[57] Nevertheless, the value of a strong basic science effort, operating in parallel to the drug discovery effort, afforded unique insights that enabled the delivery of an approved $mGlu_5$ PAM NME in less than 3 years from the initiation of the collaboration.

It is important to note that the "molecular switch" phenomenon can also be beneficial.[14,49] We and others have reported numerous examples where a small structural modification to a core has engendered unique, desired pharmacology or afforded access, through a change in subtype selectivity, to ligands for a receptor subtype that were previously unavailable.[58−63] Figure 8 shows three recent examples of the latter. Taking advantage of the promiscuity of MPEP site $mGlu_5$ PAM ligands, we noted that $mGlu_5$ PAM **30** ($mGlu_5$ EC_{50} = 270 nM) had weak activity as an $mGlu_3$

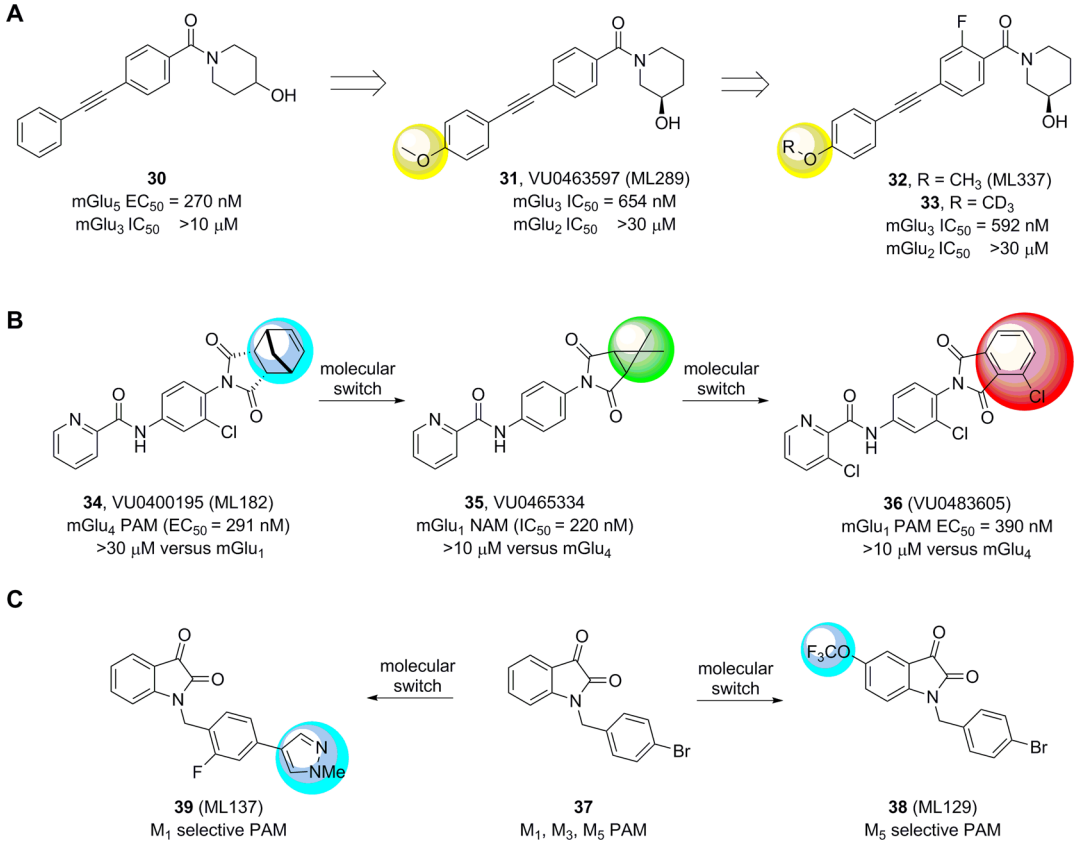

Figure 8. Beneficial "molecular switches". (A) Delivery of the first highly selective $mGlu_3$ NAM **31** by virtue of a 4-OMe "molecular switch" on an $mGlu_5$ PAM scaffold. Subsequent optimization and "fluorine walk" provided **32**, which was further optimized for DMPK properties by deuterium incorporation, as in **33**, to overcome "flat" SAR. (B) The allosteric sites on $mGlu_4$ and $mGlu_1$ historically cross-talk, and taking advantage of a "double molecular switch", an $mGlu_1$ PAM with properties suitable for in vivo studies resulted. (C) An M_1 HTS identified **37** as an M_1, M_3, M_5 PAM. A "molecular switch" in the form of a 5-OCF_3 group engendered selectivity for M_5, while addition of small heterocycles to replace the 4-Br moiety afforded selective M_1 PAMs.

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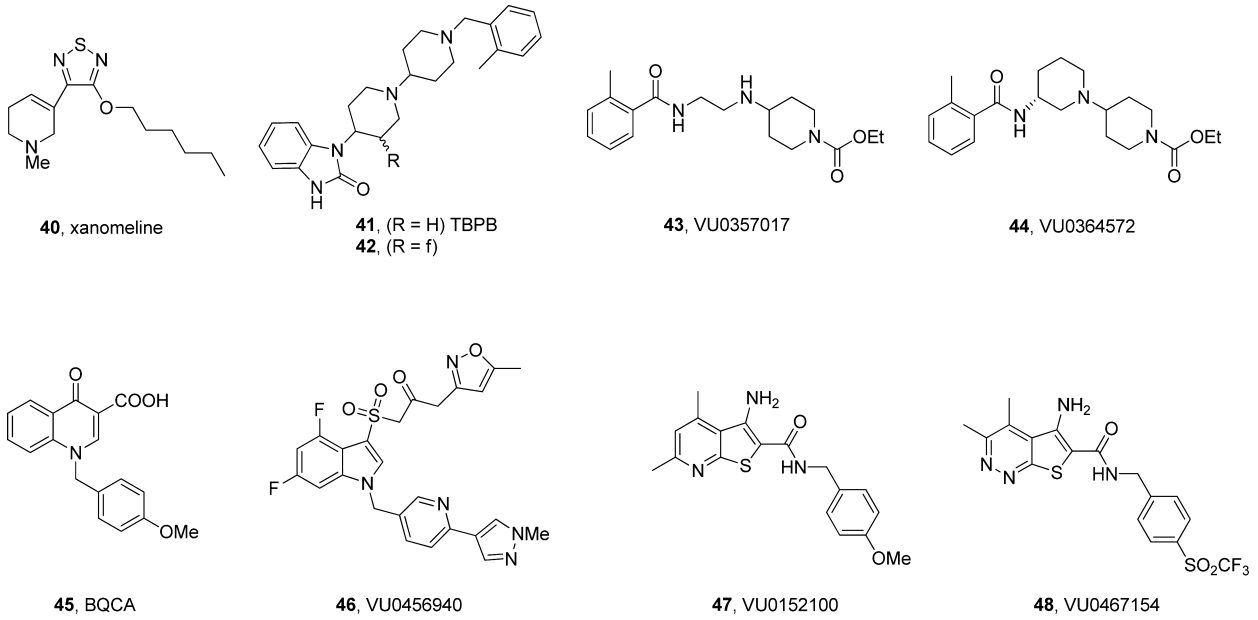

Figure 9. In pursuit of a better xanomeline. Shown are structures of xanomeline (**40**) and subsequent M_1 allosteric agonists **41**–**44**, which demonstrated that all were bitopic with receptor reserve-dependent pharmacology (e.g., brain region-specific activity) and thus not tractable as a therapeutic approach. PAMs of either M_1 (**45** and **46**) or M_4 (**47** or **48**) have emerged as important in vivo tools to demonstrate that this mode of pharmacology is superior to allosteric agonism with true mAChR selectivity.

NAM ($mGlu_3$ IC_{50} > 10 μM) and felt this could be exploited to access the first selective $mGlu^3$ NAMs.[64] A diversity-oriented synthesis effort identified a "molecular switch", again in the 4-position of the distal phenyl ring, in the form of a methoxy moiety. SAR was "flat" as anticipated, yet we were able to access VU0463597 (**31**, ML289) as the first highly selective $mGlu_3$ NAM ($mGlu_3$ IC_{50} = 650 nM, $mGlu_2$ IC_{50} > 10 μM), which was devoid of $mGlu_5$ activity (both PAM and NAM).[64] Further lead optimization and utilization of the "fluorine walk" provided the more selective $mGlu_3$ NAM probe VU06024017 (**32**, ML337); however, the key moiety that engender activity at $mGlu_3$, the 4-methoxy group, was also the lone metabolic liability, undergoing a rapid oxidative dealkylation to an inactive congener.[65] Steric and electronic modification to both the ether and the pendent aromatic ring led to a significant diminution in potency. Ultimately, this led to the introduction of another valuable tool in the allosteric modulator tool box: deuterium incorporation and reliance on the stronger C–D bond. Replacement of the OCH_3 with a OCD_3 (**33**) maintained the pharmacological profile, yet reduced in vitro and in vivo clearance by ∼50%, enabling **33** to serve as a valuable in vivo tool to study selective $mGlu_3$ inhibition.[65]

Likewise, many $mGlu_4$ PAMs and $mGlu_1$ NAMs are known in the literature, but there are few $mGlu_1$ PAMs and none with properties suitable for in vivo studies.[66] It is well established since the first report of (−)-PHCCC as an $mGlu_4$ PAM (and weak $mGlu_1$ NAM) that there was cross-talk between the $mGlu_4$ and $mGlu_1$ allosteric sites.[67] However, optimization efforts were able to separate these activities delivering highly selective $mGlu_4$ PAMs, such as **34**.[68] Further chemical optimization and modification to the imide moiety provided **35**, an equally potent and highly selective $mGlu_1$ NAM via a "molecular switch".[69] Replacement of the imide in **35** with a phthalimide, as in **36**, induced another "molecular switch", in essence a "double molecular switch" from **34**, leading to a highly selective $mGlu_1$ PAM, with properties enabling dissection of an emerging role of *GRM1* mutation in schizophrenia as well as in vivo studies (Figure 8B).[70]

Finally, this is not restricted to class C GPCRs or mGluRs. The phenomenon of "molecular switches" is prevalent across all families of GPCRs, where it has been instrumental in the development of highly subtype selective muscarinic acetylcholine (mAChR) ligands (Figure 8C). For instance, an HTS to identify M_1 PAMs generated a unique hit: a pan-G_q coupled, M_1, M_3, M_5-triple PAM **37** with equivalent low micromolar activity across the three subtypes.[71,72] A matrix library (9 × 12), surveying nine functionalized isatins and 12 different benzyl moieties, uncovered a 5-OCF_3 moiety as "molecular switch" that provided the first highly selective M_5 PAM, **38** (ML129).[71] Further optimization with iterative libraries and the "fluorine walk" afforded an *N*-Me pyrrole as a "molecular switch" leading to a highly selective M_1 PAM, **39** (ML137).[72] Both of these findings led to further optimization programs and high quality in vivo tools to probe M_1 and M_5 function in the CNS.

Before leaving GPCRs, it is important to discuss a related pharmacological approach that is often confused with PAMs: allosteric agonism. While PAMs require the orthosteric agonist and potentiate its activity, an allosteric agonist binds at a site distinct from the orthosteric site and is capable of activating the receptor in the absence of the orthosteric ligand.[2−12] This approach has been largely isolated to the M_1 mACh receptor, based on the clinical proof of concept studies with xanomeline (**40**), an M_1/M_4 preferring agonist, in patients with either Alzheimer's disease or schizophrenia (Figure 9).[73] However, like all mAChR M_1 agonists developed in the 1990s, the true lack of subtype selectivity led to activation of peripheral M_2/M_3 receptors and adverse events precluded further development, despite efficacy noted in PhII trials.[2−12] These data led to the quest for selective M_1 and/or M_4 allosteric agonists. Many weak partial and functionally selective M_1 allosteric agonists were disclosed, and a prototypical example is TBPB (**41**);[74] yet **41** and related congeners were found to be bitopic, meaning the ligand bound to an allosteric site on M_1 that led to functionally

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selective M_1 activation, but when screened in antagonist mode, they were full antagonists of the M_2−M_5, and in competition binding studies, as the concentration was increased, the orthosteric radioligand, [^{3}H]NMS was fully displaced.[60,74−76] Therefore, the "allosteric agonists" bind with high affinity at an allosteric site, but all also bind at the orthosteric site. This was further supported by modulating the basicity of the central piperidine ring of **41** with the β-F congener **42** and disrupting a key hydrogen bind at the allosteric site that led to pan-mAChR (M_1-M_5) antagonism.[76] Finally, we demonstrated that two additional partial M_1 allosteric agonists **43** and **44**[75] displayed receptor reserve-dependent pharmacology in native brain tissue preparations that translated into in vivo behavioral outcomes.[77] Thus, although there was early optimism with the approach, it became clear that allosteric agonists were actually a "second coming" of the M_1 agonists of the 1990s, laden with unpredictable activity across brain regions and susceptibility to peripheral M_2/M_3 issues due to high receptor reserve and bitopic binding/pharmacology.[77] Therefore, after years of effort and dedicated basic science, it became clear that M_1 (e.g., **45** and **46**)[78,79] and M_4 PAMs (e.g., **47** and **48**)[80,81] would be the desired pharmacological approach to achieve true mAChR subtype selectivity and advance into the clinic to validate the clinical data with **40**. Preclinically, it is clear that M_4 plays a larger role as an antipsychotic agent with modest cognition enhancing activity, whereas the reverse is true for M_1.[78−81] A program we initiated at Merck provided the first reported M_1 PAM, BQCA (**45**), where all the principles discussed (iterative parallel synthesis, matrix libraries, and the "fluorine walk") were critical in lead optimization and ultimately led to the development of MK-7622 (structure not officially disclosed) which is currently in phase II clinical trials, and efficacy data are eagerly awaited.

Finally, our group has contributed important small molecule allosteric modulators for other GPCRS including $mGlu_1$ (PAMs and NAMs), $mGlu_3$ (NAMs), $mGlu_4$ (PAMs and NAMs), $mGlu_5$ (PAMs and NAMs), pan group III mGluRs (PAMs), M_1 (PAMs and allosteric agonists), M_4 (PAMs), M_5 (PAMs and NAMs), and even class B GPCRs such as GLP-1 (PAMs).[2−12,14] These programs gave rise to the principles outlined in Chart 1 and referred to throughout this Perspective. While targeting allosteric sites has provided highly selective druglike compounds with good CNS exposure to understand the physiological underpinnings and therapeutic potential of many discrete GPCRs, it is not a panacea. Judicious evaluation of species differences must be adhered to along with the "triple add" primary screen to capture molecular switches, which carries forward into DMPK to understand the pharmacology of metabolites. Furthermore, stimulus bias can be beneficial as well as harmful; therefore, pharmacological characterization across a broader panel of assays to assess stimulus bias must be performed on HTS lead series and prior to focusing on a given scaffold. Although insights from mutagenesis work as to the origin of "molecular switches" have been helpful, the surge of new X-ray crystal structures of relevant GPCRS, some with allosteric ligands bound,[82] is very exciting and will hopefully pave the way for a greater understanding of allosteric SAR.

IV. ALLOSTERIC MODULATION OF PHOPSPHODIESTERASES: THE CASE OF PHOSPHOLIPASE D (PLD)

Phospholipase D (PLD) is a phospholipase that catalyzes the production of choline and phosphatidic acid (PA), an important lipid second messenger involved in a myriad of critical signaling and metabolic pathways by the hydrolysis of phosphatidylcholine.[83] Mammals possess two isoforms of PLD, termed PLD1 and PLD2 (sharing 53% homology), both of which are differentially regulated and possess independent physiological roles. Structurally, PLD, like Akt, is a highly flexible enzyme that consists of a highly conserved active site (composed of two histidine-lysine-aspartate amino acid domains) and both N-terminal phox homology (PX) and PH regulatory domains.[83] Extensive biochemical data and genetic studies have implicated dysfunction in PLD signaling and/or PLD overexpression in cancer, viral infection, and CNS disorders; however, the therapeutic potential of modulating PLD function remained elusive for over 20 years since its discovery because of a lack of selective and PLD isoform specific small molecules. Indeed, the field has been driven by the use of *n*-butanol (**49**), which competes for water in a transphosphatidylation reaction with water (Figure 10).[83]

A 2007 short report from Novartis described halopemide (**50**), an atypical antipsychotic agent, and a collection of 12 related congers as PLD inhibitors.[84] Upon recognition of clinical trial data with **50**, wherein both isoforms of PLD were inhibited in man with normal biochemistry and without adverse events generated great interest in a field lacking small molecule tools.[85] Because of the presence of a PH domain in PLD and the piperdine benzimidazolone moiety in **50**, we believed **50** may be inhibiting the enzyme through an allosteric mechanism, akin to Akt, and that the dual PLD1/2 inhibitor **50** could represent an attractive lead from which to access PLD1 and PLD2 selective compounds. Thus, our laboratory in collaboration with Alex Brown and his laboratory initiated a diversity-oriented synthesis campaign around **50**, followed by iterative parallel synthesis and the "fluorine walk" (Figure 10), to develop the first direct, isoform selective PLD inhibitors **51** (1700-fold PLD1 selective) and **52** (75-fold PLD2 selective).[86−88] Mutants lacking the N-terminus (PX and PH domains) lost PLD activity (again similar to Akt), and when combined with other biochemical and enzyme kinetic studies, it became clear that these compounds were indeed allosteric. Despite value in aiding to more clearly define the individual contributions of PLD1 and PLD2 in various systems and diseases, poor DMPK and physiochemical properties precluded robust in vivo studies.[86−88] Further chemical optimization led to the discovery of the potent dual PLD1/2 inhibitor ML299 (**53**) and a second generation PLD2 selective probe ML298 (**54**).[89] Both showed improved ancillary pharmacology but only marginally improved DMPK profiles and physiochemical properties. Here, we noted a "molecular switch" in the form of a "magic methyl" that enhanced PLD1 activity ∼50-fold in the piperidine benzimidazolone series represented by **51** but could reverse PLD2 selectivity over 250-fold in the triazaspirone series, represented by **53**.[89] While further optimization to improve DMPK continues, the tools in hand elucidated a wealth of information regarding the therapeutic potential of PLD inhibition, especially PLD2. For example, **52** was critical in studies that identified PLD as a novel regulator of Akt in glioblastoma multiforme (GBM).[90] **52** enabled studies that showed PA as an essential component for the membrane recruitment and activation of Akt, as well as a direct protein−protein interaction between PLD2 and Akt. Inhibition of PLD_2 by **52** decreases activation of Akt leading to cell death through inhibition of autophagic flux and a back door by which to inhibit Akt.[90] Furthermore, we demonstrated that infection by

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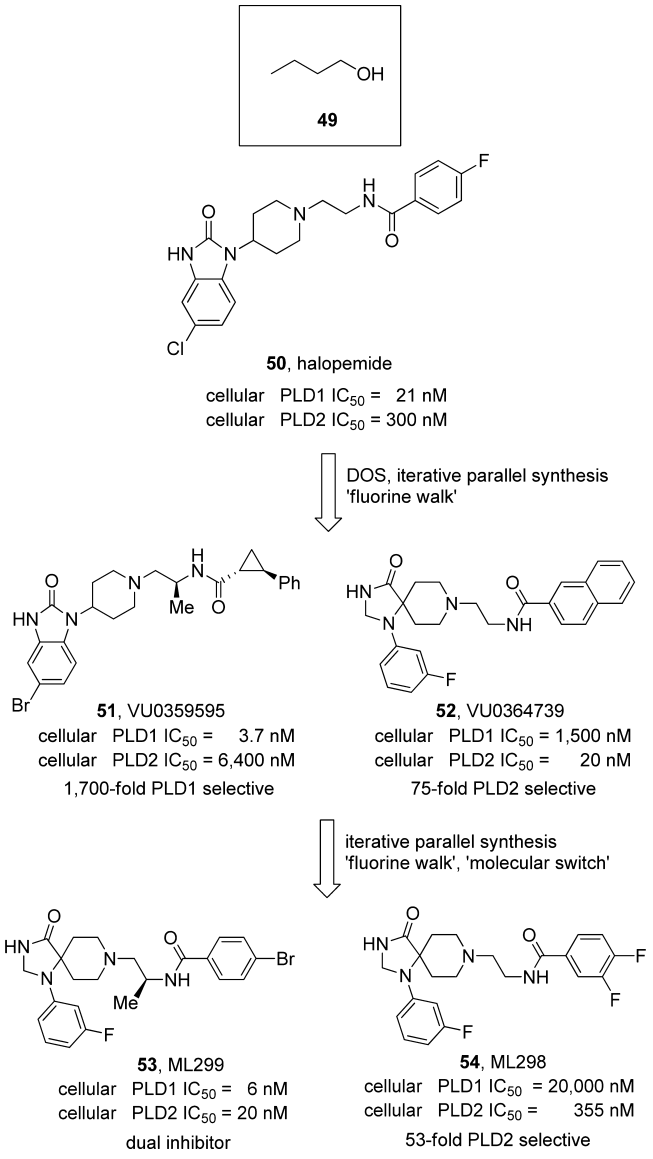

Figure 10. Development of allosteric, isoform selective PLD inhibitors **51**–**54**, utilizing DOS, iterative parallel synthesis, the "fluorine walk" and "molecular switches", all of which were borrowed from the allosteric GPCR field.

influenza virus stimulates phospholipase D (PLD) activity and PLD colocalizes with influenza during infection.[91] Inhibition of PLD2 with **52** delayed viral entry and reduced viral titers in vitro and in vivo, as well as enhancing survival against a broad panel of influenza strains (H1, H3, H5, and H7).[91] Once again, development of highly isoform selective PLD inhibitors, by targeting an allosteric mechanism, advanced our understanding of the deeper signaling biology and uncovered therapeutic potential in oncology and virology. Moreover, principles, strategies, and issues for optimization of GPCR allosteric ligands carried over into phospholipases.

V. CONCLUSION

Allosteric modulation has fundamentally altered our ability to prosecute "tough" targets and successfully develop ligands. Here, in commemoration of the 2013 Philip S. Portoghese Medicinal Chemistry Lectureship, several vignettes of drug discovery campaigns targeting novel allosteric mechanisms for kinases, phospholipases, and G-protein-coupled receptors were described that gave rise to general principles for successful optimization. On the basis of the broad applicability and success of allosteric modulation, we wish to move beyond classical drug repurposing to "receptor repurposing" and re-engage targets that failed because of the ligands/chemotypes with new functional HTS campaigns and subsequent development of allosteric ligands and exploit fundamentally new chemotypes and biased signaling profiles. Phase II clinical data are eagerly awaited for multiple allosteric modulators (Akt, M_1 PAMs, etc.) to further validate the approach to improve human health and impact unmet medical needs.

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Notes

The authors declare the following competing financial interest(s): Funding was received from BMS and AZ for allosteric modulators of mGlu4 and M4, respectively.

Biography

Craig W. Lindsley is Director of Medicinal Chemistry for the Vanderbilt Center for Neuroscience Drug Discovery in Nashville, TN, and holds the William K. Warren, Jr. Chair in Medicine. He received his doctorate in 1996 from the University of California, Santa Barbara, and pursued postdoctoral studies at Harvard University, MA. In 2001, he moved to Merck and developed a streamlined approach for lead optimizaton that resulted in the delivery of six preclinical candidates, including the first isoenzyme-selective allosteric AKT-kinase inhibitors, as well as the first positive allosteric modulators of the $mGlu_5$ and M_1. In 2006, he accepted Associate Professor appointments in Pharmacology and Chemistry at Vanderbilt University, and in 2009 he was promoted to full Professor and is the founding Editor-in-Chief for *ACS Chemical Neuroscience*.

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ABBREVIATIONS USED

CNS, central nervous system; GPCR, G-protein-coupled receptor; ATP, adenosine triphosphate; CNS, central nervous system; PAM, positive allosteric modulator; NAM, negative allosteric modulator; SAM, silent allosteric modulator; NAL, no affect ligand; BZD, benzodiazepine; $GABA_A$, γ-aminobutyric acid; mAChR, muscarinic acetylcholine receptor; ACh,

acetylcholine; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; DMPK, drug metabolism/pharmacokinetic; SAR, structure−activity relationship; GLP1, glucagon-like peptide 1; PET, positron emission tomography; CRC, concentration−response curve; FRET, fluorescence resonance energy transfer; HTS, high-throughput screen; DFB, 3,3′-difluorobenzaldazine; CDPPB, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; MPEP, 2-methyl-6-(phenylethynyl)pyridine

■ ADDITIONAL NOTE

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