

# Drugability of Extracellular Targets: Discovery of Small Molecule Drugs Targeting Allosteric, Functional, and Subunit-Selective Sites on GPCRs and Ion Channels

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Beginning with the discovery of the structure of deoxyribose nucleic acid in 1953, by James Watson and Francis Crick, the sequencing of the entire human genome some 50 years later, has begun to quantify the classes and types of proteins that may have relevance to human disease with the promise of rapidly identifying compounds that can modulate these proteins so as to have a beneficial and therapeutic outcome. This so called 'drugable space' involves a variety of membrane-bound proteins including the superfamily of G-protein-coupled receptors (GPCRs), ion channels, and transporters among others. The recent number of novel therapeutics targeting membrane-bound extracellular proteins that have reached the market in the past 20 years however pales in magnitude when compared, during the same timeframe, to the advancements made in the technologies available to aid in the discovery of these novel therapeutics. This review will consider select examples of extracellular drugable targets and focus on the GPCRs and ion channels highlighting the corticotropin releasing factor (CRF) type 1 and  $\gamma$ -aminobutyric acid receptors, and the Ca<sub>v</sub>2.2 voltage-gated ion channel. These examples will elaborate current technological advancements in drug discovery and provide a prospective framework for future drug development.

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## INTRODUCTION

The term 'drugability' (or 'druggability') has not yet achieved mainstream status enough to warrant a definition in Webster's, however, for those in the fields of genetics and medicine in drug discovery, it is a term extremely well understood. Loosely, this term refers to the ability of a xenobiotic or small molecule to modulate the function of an endogenous protein and have a beneficial effect on the organism. More specifically, this term relates to drug discovery and the ability of modifying a disease state through a specific protein interaction or mechanism in the body. The methodologies for drug discovery have enjoyed a rapid evolution in a relatively short period time from the initial direct radioligand-binding experiments designed to test single molecular entities, to sophisticated technologies that allow large chemical libraries to be synthesized and tested on multiple targets simultaneously using high-throughput liquid handling and detection techniques. These advancements in technology,

however, have not yet yielded that expected quantum leap in available therapeutics for unmet medical needs. Leaving aside for a moment the increasing hurdles of safety, development, and regulatory environments that all potential drugs face, the initial selection of a target, and the assessment of its drugability as a disease-relevant molecular mechanism is paramount in the discovery of novel therapeutics.

The sequencing of the human genome in 2001 by the International Human Genome Sequencing Consortium estimated in draft form, that the genome contains approximately 30 000 genes (Lander *et al*, 2001; Venter *et al*, 2001). This collaboration later converted this draft to a final result reducing the initial number of gaps from ~150 000 to 400 resulting in a refinement of the estimates to a genome containing between 20 000 and 25 000 genes (Consortium, 2004). Of these, estimates range from 10 to 15% of these genes that can generate drugable targets and only half of those may be truly related to proteins that are actually linked to disease (Hopkins and Groom, 2002). Of the myriad of proteins that are expressed on the surface of cells, some entirely specific to the type of cell in the body, two families lead the drugable universe. These two families are the G-protein-coupled receptors (GPCRs); proteins that are involved in transducing signals from the outside of the

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cell to the inside of the cell through endogenous ligands, and the ion channels; proteins that either through endogenous ligands or electrically evoked signals, modulate the membrane potential of cells by allowing the transport of ions through the extracellular membrane. On the basis of the information gained from the description of the human genome previously described, GPCRs represent the largest drugable target family (represented by 1000–2000 genes) and are currently the targets for 30–40% of the drugs on the market today (Hopkins and Groom, 2002). Ion channels appear to be represented by 400 genes in the human genome (Venter *et al*, 2001) and are currently the targets for approximately 7% of the current marketed drugs (Hopkins and Groom, 2002). Even with these impressive statistics, a significant portion of identified GPCRs and ion channels still remain to be characterized, and more importantly, their relevance to specific disease states elucidated. To complicate matters somewhat further, there is still a popular notion that within the human genome, there are a finite number of drugable targets specifically defined by these genes (one gene = one target). However, the future prospects are much more sanguine when we explore some of the novel technological advances that are continually being developed.

The elucidation of multimeric proteins formed in response to disease, allosteric interactions of small molecules, cell-specific signaling mechanisms, and biased agonism (the selective activation of specific signaling pathways through the same receptor protein dependent on differential ligand/receptor interactions) all offer opportunities to evaluate the drugable space from different perspectives, in fact, increasing the number, scope and definition of 'drugable' targets beyond the one-gene-per-target approach. In a very recent example of biased agonism, Drake *et al* (2008) have shown, that selective ligands for the  $\beta_2$ -adrenergic receptor can cause differential intracellular signaling by stimulating  $\beta$ -arrestin-dependent receptor activities to a much greater extent than their given efficacy for G-protein activation leading to a potential for development of a novel class of receptor modulators. This paper will focus on the characteristics of two families of extracellular drugable targets, the GPCRs and ion channels, and illustrate some of the challenges and the methodologies and advancements used to overcome some of these challenges that have led to the successful discovery of small molecule therapeutics in disease; some currently in clinical trials.

## G-PROTEIN-COUPLED RECEPTORS

### Drug Development Challenges for Newly Discovered G-Protein-Coupled Receptors

Early drug discovery on GPCRs in central nervous system (CNS) diseases focused on small molecule receptors, typified by the monoamine receptors. These receptors were immediately tractable drug targets largely because their binding pockets have evolved to bind their small, drug-like

endogenous ligands. These ligands, such as dopamine, norepinephrine or serotonin, provided ideal chemical starting points for drug development. With the application of molecular biology, the diversity of molecular targets in CNS diseases expanded enormously. Many of the newly discovered GPCR targets were quite different from the classical monoamine GPCRs, both in terms of the molecular structure of the receptors themselves and the size of the endogenous ligands that activate them. These two features presented a considerable challenge to developing drug-like molecules for the receptors. In this section, it will be shown how an understanding of the structural and mechanistic basis of receptor binding and activation has been applied to improve the 'drugability' of these targets resulting in the successful development of drug-like molecules.

### GPCR Structure and Function: Allosteric Modulation in Drug Development for Complex GPCRs

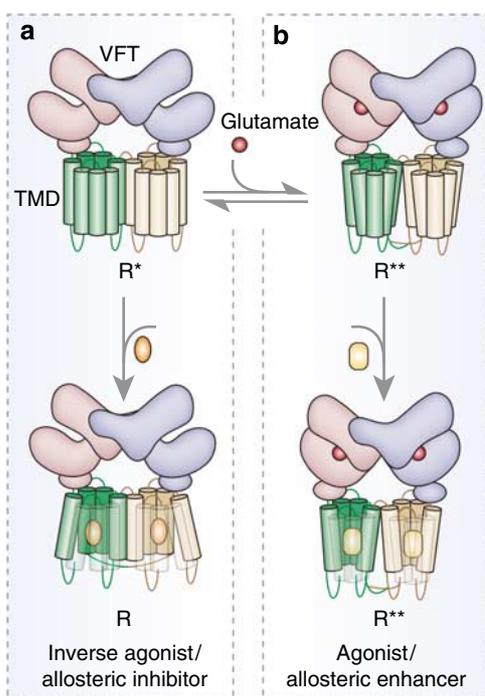
GPCRs for small CNS ligands, such as the monoamines epinephrine and dopamine, are generally straightforward drug targets. These GPCRs are comprised of a single domain of seven membrane-spanning  $\alpha$ -helices that both binds the ligand and propagates the signal of G-protein activation (Ballesteros *et al*, 2001). The binding site has evolved to bind a small ligand, formed as a pocket within the cluster of  $\alpha$ -helices, and so is tractable for small molecule medicinal chemistry (Rasmussen *et al*, 2007). However, not all small CNS ligands bind simple GPCRs. The excitatory neurotransmitter glutamate and the inhibitory  $\gamma$ -aminobutyric acid (GABA), besides acting on ion channels to directly mediate neurotransmission, also modulate GPCRs in the CNS (Heresco-Levy, 2005; Niswender *et al*, 2005). These GPCRs present a considerable challenge to drug development owing to a complicated receptor structure. When the first glutamate GPCR was cloned in 1991 (Houamed *et al*, 1991; Masu *et al*, 1991), it was found to possess a strikingly different primary structure from the GPCRs cloned previously, revealing a new GPCR subfamily, termed the class C GPCRs. The predicted structure possessed seven membrane-spanning  $\alpha$ -helices, like all GPCR's, but the predicted N-terminal extracellular domain (ECD) was much larger (hundreds of amino-acid residues), raising the question of how the receptor bound glutamate and was activated. The second unusual feature of this GPCR family was revealed when the GABA<sub>B</sub> receptor was cloned (Bettler *et al*, 2004). Previously, the GABA<sub>B</sub> receptor had been defined pharmacologically as the receptor for the antispastic drug baclofen. When initially cloned and expressed, the pharmacological profile of the cloned receptor did not fully recapitulate the expected activity of a GABA<sub>B</sub> receptor and the signaling efficacy of the receptor was surprisingly low (Kaupmann *et al*, 1997). Subsequently, a number of groups demonstrated a surprising phenomenon. A second subtype of the receptor was identified and when the two receptors were coexpressed a fully functional

GABA<sub>B</sub> receptor was finally demonstrated to be a dimer of the two subunits (Kaupmann *et al*, 1998; Ng *et al*, 1999; White *et al*, 1998). The dimer concept was also demonstrated for other members of the class C family, such as the metabotropic glutamate receptors and the calcium-sensing receptor (Pin *et al*, 2005).

The unusually large extracellular region is comprised of a Venus fly-trap (VFT) domain, revealed by X-ray crystallography (Kunishima *et al*, 2000; Tsuchiya *et al*, 2002) that binds the endogenous ligand (glutamate or GABA). In an inactive state the lobes of this domain are separated in an open configuration (Figure 1a). In the active state of the VFT, promoted by binding of an endogenous ligand (Figure 1b), the lobes are brought together in a closed configuration (Kunishima *et al*, 2000; Pin *et al*, 2005; Tsuchiya *et al*, 2002). The VFT is also involved in dimer formation between the two subunits that comprise the receptor (Pin *et al*, 2005); a key feature in the basic understanding of the functional nature of this particular protein. The VFT is connected to the transmembrane

domain (TMD) comprising the seven membrane-spanning  $\alpha$ -helices (Figure 1). The TMD, mediating G-protein activation, can exist in inactive and active conformations (Parmentier *et al*, 2002), with three predominant conformations being proposed—inactive, partially active, and fully active (Pin *et al*, 2005), designated here as R, R\*, and R\*\*, respectively (Figure 1). The conformation of the TMD is subsequently modulated by the VFT. This structure of class C GPCRs presented both a problem and an opportunity for drug development. The first apparent problem was simply a technical one for the GABA<sub>B</sub> receptor. The discovery and elucidation of GABA<sub>B</sub> receptor heterodimerization allowed the expression of a fully functional cloned receptor for use in drug discovery and development (Bettler *et al*, 2004). The availability of the cloned receptor allows pharmacological characterization of a specified molecular target, rather than an activity in a tissue. The structure of the cloned receptor can also be modified using the techniques of molecular biology to introduce specific amino-acid mutations allowing a detailed investigation of direct structure–function relationships that can aid compound SAR. This enabled modern drug discovery utilizing these cloned and expressed receptors in high-throughput *in vitro* testing, both for lead discovery through screening (HTS) and for lead optimization through focused medicinal chemistry. The second problem is that the structural organization of class C GPCRs is much more complex than the monoamine GPCRs; the ligand-binding site and G-protein activation region reside on different domains of the receptor and, in the case of the GABA<sub>B</sub> receptor, the heterodimer of two GPCR subunits is required for receptor function. The application of this new knowledge of receptor structure and function to aid drug discovery will be detailed below.

Allosteric modulation represents one potentially tractable approach for drug development on class C GPCRs. Allosteric modulators are compounds that bind sites on the receptor that are spatially distinct from the endogenous ligand-binding site (Birdsall *et al*, 1995; Christopoulos, 2002). Put simply, the multiplicity of functional domains of class C GPCRs provides multiple sites for drugs to target beyond a direct action on the endogenous ligand-binding cleft on the VFT. Furthermore the conformational regulation inherent in signal transduction by the endogenous ligand can be coopted in allosteric regulation by pharmacological agents (Niswender *et al*, 2005; Pin *et al*, 2005). The TMD has been exploited as a drugable allosteric site for class C GPCRs, especially metabotropic glutamate receptors (Rudd and McCauley, 2005). This domain is tractable for drug development presumably because the membrane-spanning  $\alpha$ -helices are arranged to form a pocket that can bind small molecules, like monoamine receptors. This idea is supported by the finding that the TMD expressed in isolation binds the small molecule ligands (Binet *et al*, 2004; Goudet *et al*, 2004). A variety of functional activities and allosteric mechanisms of these molecules have been identified (reviewed in detail in Parmentier *et al*, 2002). In the simplest mechanisms, compounds can bind the TMD



**Figure 1.** Diagrammatic representation of the domain structure of class C GPCRs. The receptor is comprised a dimer of subunits. Each subunit is divided into the bi-lobed Venus fly-trap (VFT) domain, which binds the endogenous ligand, connected to the transmembrane domain (TMD), which activates G-protein (Pin *et al*, 2005). The structure of the VFT has been solved (Kunishima *et al*, 2000; Tsuchiya *et al*, 2002). The TMD has been proposed to adopt multiple conformational states differing in their extent of activation (Pin *et al*, 2005), denoted here as the inactive state R, the partially active state R\*, and the fully active state R\*\*. Panel (a) shows that allosteric inhibitors bind the TMD and promote the inactive state R, attenuating activation by the endogenous ligand. Panel (b) shows that allosteric enhancers bind the TMD and stabilize the fully active conformation R\*\* that enhances the activity of the endogenous ligand. More complex modes of allosteric regulation have also been demonstrated on class C GPCRs (Parmentier *et al*, 2002).

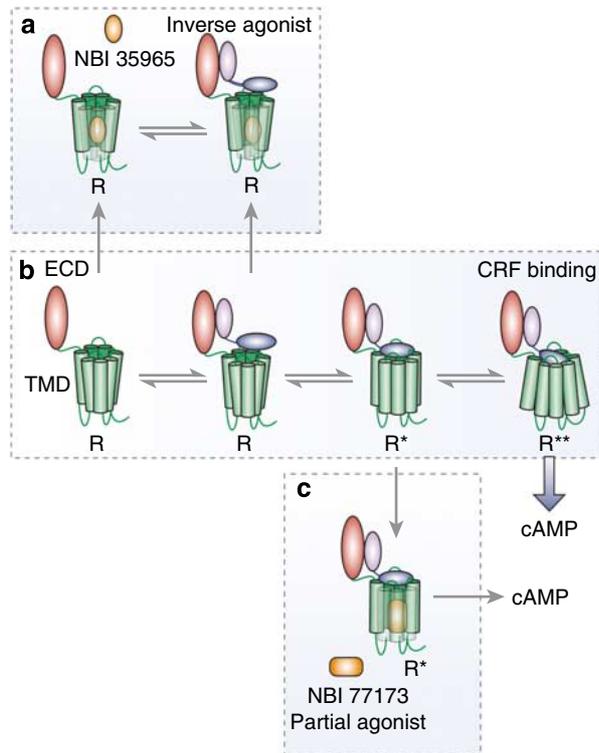
and activate it (agonism, Figure 1b), or bind this domain and prevent it from signaling (inverse agonism, Figure 1a). Agonism results from the small molecule stabilizing and promoting the formation of an active state ( $R^{**}$ ) of this domain (Figure 1b), although the presence of the VFT might hinder this process (Binet *et al*, 2004; Goudet *et al*, 2004; Pin *et al*, 2005). Reciprocally, inverse agonism results from small molecule promoting an inactive state of the transmembrane (TM) region (R; Goudet *et al*, 2004; Figure 1a). These ligands can also modulate activity of the endogenous ligands. Stabilization of R by inverse agonists (allosteric inhibitor) blocks the signaling activity of endogenous ligand, and agonist stabilization of the  $R^{**}$  state (allosteric enhancer) results in potentiation of signaling by endogenous ligand (Parmentier *et al*, 2002; Pin *et al*, 2005; Figure 1). More complex modes of allosteric regulation have also been identified. For example, GABA binds the VFT of the  $GABA_{B1}$  subunit, which in turn activates, *in trans*, the TMD of the  $GABA_{B2}$  subunit within the heterodimer (Pin *et al*, 2005). In addition, there is a functional interaction between the VFT and TMDs that can be modulated by a ligand. The presence of the VFT suppresses direct activation of the TMD by allosteric ligands, however, these ligands potentiate the activation of the TMD by the activated VFT. This type of modulation (silent allosteric enhancement) allows allosteric ligands to have little activity by themselves but to potentiate the activity of the endogenous ligand.

Overall, understanding the allosteric mechanism of action of class C GPCR modulators has been critical in evolving initial leads, identified from HTS (Johnson *et al*, 2003), through lead optimization medicinal chemistry efforts to compounds that are currently being tested clinically (Niswender *et al*, 2005). Examples of these compounds include cinacalcet (compound 1, Figure 4a), an allosteric enhancer of the calcium-sensing receptor used to treat hypercalcemia associated with renal failure (Harrington and Fotsch, 2007), LY487379 (compound 2, Figure 4a) and other allosteric enhancers of group II mGlu receptors developed as potential treatments for schizophrenia (Rudd and McCauley, 2005), and CGP7930 (compound 3, Figure 4a) along with other  $GABA_B$  receptor potentiators for treating addiction (Adams and Lawrence, 2007). The structural complexity that has led us down the path of allosteric modulation may also provide therapeutic value. As discussed extensively elsewhere, allosteric modulation maintains the spatial and temporal limits of endogenous receptor activation; receptor activity is modulated only where and when the endogenous ligand is present (Birdsall *et al*, 1995; Christopoulos, 2002). In contrast, effective concentrations of direct-acting agonists stimulate the receptor wherever the agonist is located and throughout the duration of agonist exposure, potentially leading to overstimulation of the target receptor and overdose, receptor desensitization in the target tissue, or side effects in collateral tissues. Silent allosteric enhancement, an extreme example of this phenomenon that is not easily permissible with the more straightforward receptor organi-

zation of a monoamine GPCR, is crucial for the safe activity of the marketed drug cinacalcet on the calcium-sensing receptor. This drug acts in concert with calcium ions to activate this receptor, potentiating the negative feedback loop of blood calcium regulation, preventing potentially fatal hypercalcemia in renal failure. As the drug does not activate the calcium-sensing receptor alone, it does not lower blood calcium to dangerous levels thus providing the beneficial effect with limited risk. Allosteric modulation may also aid development of compounds that target specific receptor subtypes that all bind the same endogenous ligand, especially for specific subtypes of the family of eight metabotropic glutamate receptors (Rudd and McCauley, 2005). The endogenous ligand-binding site must preserve some structural similarity through evolution of receptor subtypes to bind the same endogenous ligand (the glutamate-binding domain on the VFT of mGlu receptors) whereas no such evolutionary pressure exists to preserve the structure of novel drugable sites (such as the discrete areas within the TMD).

Allosteric modulation has also been exploited for another family of GPCR's, the class B GPCR family (Foord *et al*, 2002; Hoare, 2005). These receptors have been traditionally viewed as particularly difficult to target with small molecules because of two potential complications, the structure of the receptor (discussed here) and the nature of the ligand (moderately sized peptides of 30–40 amino-acid residues, see below; Hoare, 2005). From a therapeutic perspective, one of the most interesting class B GPCRs in the CNS is the corticotropin releasing factor (CRF) type 1 receptor. Central  $CRF_1$  receptors have been rationalized as a potential novel target for the treatment of mood disorders including depression and anxiety (Grigoriadis, 2005; Hauger *et al*, 2006; Holsboer, 2003). This proposal is based on the hypothesis that anxiety and depression are stress-related disorders and the established role of CRF as a principal regulator of the stress axis: CRF is a 41 amino-acid peptide that acts by the pituitary to regulate the hypothalamic–pituitary axis and in the CNS to modulate behavioral responses to stress (Bale and Vale, 2004; Smagin and Dunn, 2000).

The structure and mechanism of class B GPCRs, such as the  $CRF_1$  receptor, is more complex than the simple monoamine receptors, although not as daunting as the complexity of class C GPCRs. The receptors comprise a TMD formed of seven membrane-spanning  $\alpha$ -helices, and a moderately sized ECD of about 100–160 amino-acid residues (Figures 2 and 3). Like class C receptors, the ECD binds the endogenous ligand and the TMD activates G-protein, but the binding mechanism is slightly different because the peptide ligands need to bind to both the ECD and the TMD of class B GPCRs (Hoare, 2005). According to this 'two-domain' model, elaborated in Figure 2b for the  $CRF_1$  receptor, the C-terminal portion of the peptide binds the ECD of the receptor (Grace *et al*, 2004; Perrin *et al*, 1998). This 'affinity-trap' interaction brings the N-terminal portion of the peptide into the vicinity of the TMD,



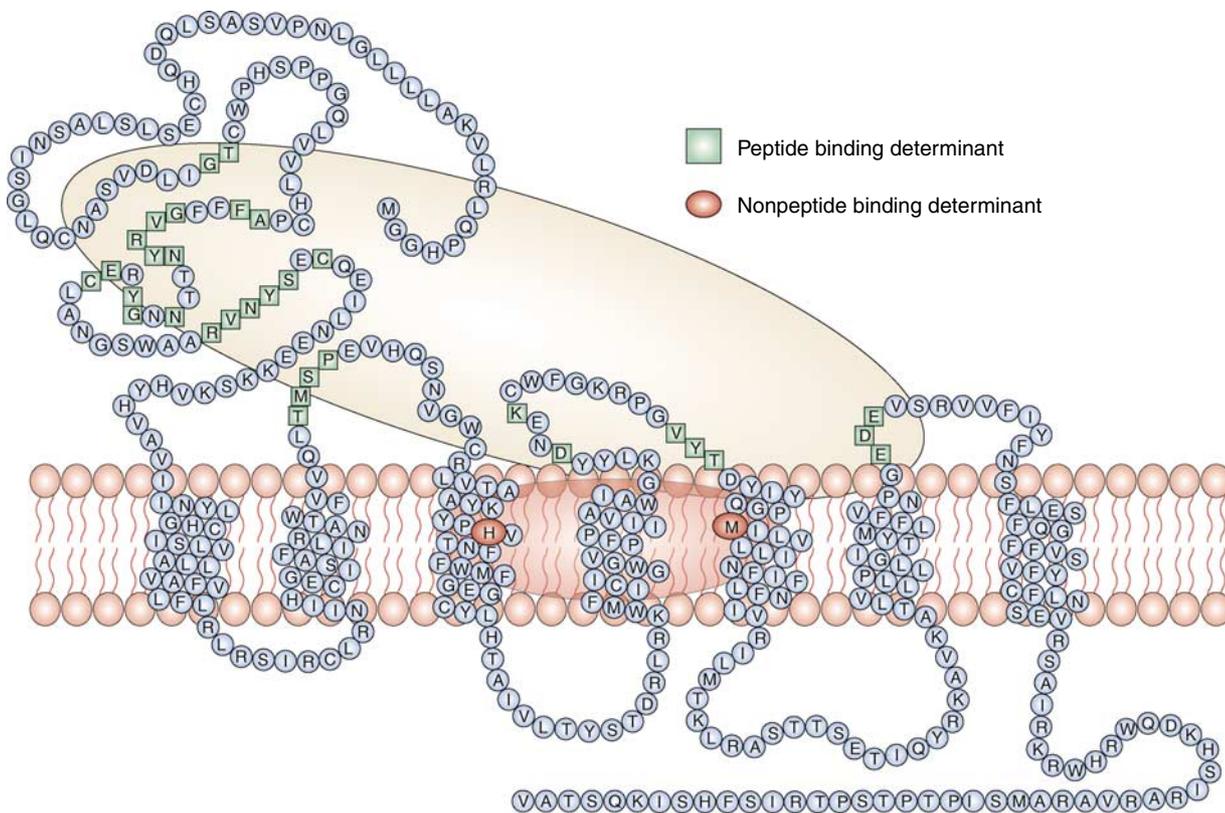
**Figure 2.** Schematic representation of CRF<sub>1</sub> receptor activation by CRF and antagonism by small molecules. The CRF-binding mechanism is illustrated in panel (b). The C-terminal portion of CRF (purple) binds the N-terminal extracellular domain (ECD) of the receptor. This ‘affinity-trap’ interaction brings the N-terminal region of the peptide (blue) into the vicinity of the transmembrane domain (TMD) of the receptor. Interaction of the N-terminal CRF portion with the TMD results in transition of the TMD from an inactive state (R) to a weakly active state (R\*), and to a fully active state (R\*\*). Panel (a) shows the mechanism of action of a standard CRF<sub>1</sub> receptor antagonist, NBI 35965. This compound binds the TMD and locks it in the inactive state R. Once NBI 35965 is bound, CRF can still bind the ECD but CRF interaction with the TMD is inhibited because the receptor is stabilized in the inactive state R. Panel (c) shows the activity of the unusual CRF<sub>1</sub> receptor weak partial agonist NBI 77173. This compound preferentially binds and stabilizes the weakly active state R\*. The model is built from data in Dautzenberg *et al* (1998), Grace *et al* (2004), Hauger *et al* (2006), Hoare *et al* (2003), (2004), (2008), Liaw *et al* (1997a, b), Mesleh *et al* (2007) and Perrin *et al* (1998).

facilitating this second interaction (Grace *et al*, 2004; Hauger *et al*, 2006; Hoare *et al*, 2004). Binding of the N-terminal peptide region to the TMD effects a conformational change to the protein that activates this receptor region, resulting in G-protein recruitment and subsequent intracellular signaling (Hoare *et al*, 2004; Nielsen *et al*, 2000). This activation process likely involves two distinct active conformations of the TMD (Figure 2b), the fully active state R\*\* that is the principle driver of G-protein activation, and a weakly active state R\* that detectably signals only when the receptor is highly over expressed (Hoare *et al*, 2008). Interestingly, the two domains of the CRF<sub>1</sub> receptor are functionally independent; peptide ligands bind isolated ECD fragments (Grace *et al*, 2004; Perrin *et al*, 1998), and are able to stimulate signaling through isolated TMD fragments (Hoare *et al*, 2004; Nielsen *et al*, 2000). This

two-domain model has been elaborated in detail for other members of the class B GPCR family (reviewed in Hoare, 2005), although a partially different model has been proposed for the secretin receptor (Dong *et al*, 2006).

As was the case for the class C GPCRs, the complexity of class B receptor structure and mechanism provides an opportunity for the development of allosteric modulators, since more than one domain on the receptor can be targeted, and the endogenous ligand interaction involves conformational modulations than can be exploited by small molecules. Compounds that block CRF signaling on the CRF<sub>1</sub> receptor in the CNS have been developed as potential treatments for depression and anxiety. The compounds were initially identified through HTS, were subsequently developed using medicinal chemistry (see below) and have since progressed into clinical trials (Binneman *et al*, 2008; Ising *et al*, 2007; Zobel *et al*, 2000). These compounds (see Figure 4e) are all allosteric modulators of the CRF<sub>1</sub> receptor (Hoare *et al*, 2003). The binding determinants for the compounds are located centrally within the TMD (Figure 3; Hoare *et al*, 2006; Liaw *et al*, 1997a), spatially separated from the binding determinants for CRF, which are located in extracellular-proximal regions of the receptor (the extracellular face of the TMD and within the ECD, Figure 3, (Dautzenberg *et al*, 1998; Hauger *et al*, 2006; Hoare *et al*, 2006; Liaw *et al*, 1997a, b). Binding of standard chemotype small molecules within the TMD stabilizes the TMD in the inactive state R (see Figure 2a), such that these compounds are classified as inverse agonists (Hoare *et al*, 2008). Stabilization of this inactive state inhibits binding of CRF to the TMD (Hoare *et al*, 2004; Nielsen *et al*, 2000). Consequently, the CRF–receptor interaction required to activate the receptor is blocked, so the compounds inhibit CRF signaling (Figure 2a). Interestingly, a structurally unusual small molecule NBI 77173 has been shown to block the CRF<sub>1</sub> receptor through a different mechanism (Hoare *et al*, 2008). Instead of stabilizing the inactive state of the TMD, this compound stabilizes the weakly active state R\* (Figure 2c). This difference is evidenced by NBI 77173 eliciting partial agonism rather than inverse agonism of a highly amplified cAMP response by a highly expressed receptor. This stabilization counteracts transition to the fully active state R\*\* that would be stimulated by CRF, blocking transition to the receptor state that is the main driver of G-protein activation, resulting in antagonism of signaling (Figure 2c). Targeting an alternative conformation of the CRF<sub>1</sub> receptor, such as the weakly active state instead of the inactive state, could facilitate the development of novel compounds with different chemical structure. Although these mechanisms have been elaborated in detail for the CRF<sub>1</sub> receptor, it is not presently clear whether they are operational for other class B GPCRs. However, we have postulated that small molecules acting on other peptide class B GPCRs are likely to act in an allosteric manner (Hoare, 2007).

From a purely structural perspective, the molecular biology revolution revealed a broad diversity of GPCRs of



**Figure 3.** Location of peptide and small molecule-binding determinants on the CRF<sub>1</sub> receptor. See Refs. Dautzenberg *et al* (1998), Hauger *et al* (2006), Liaw *et al* (1997a, b) for details.

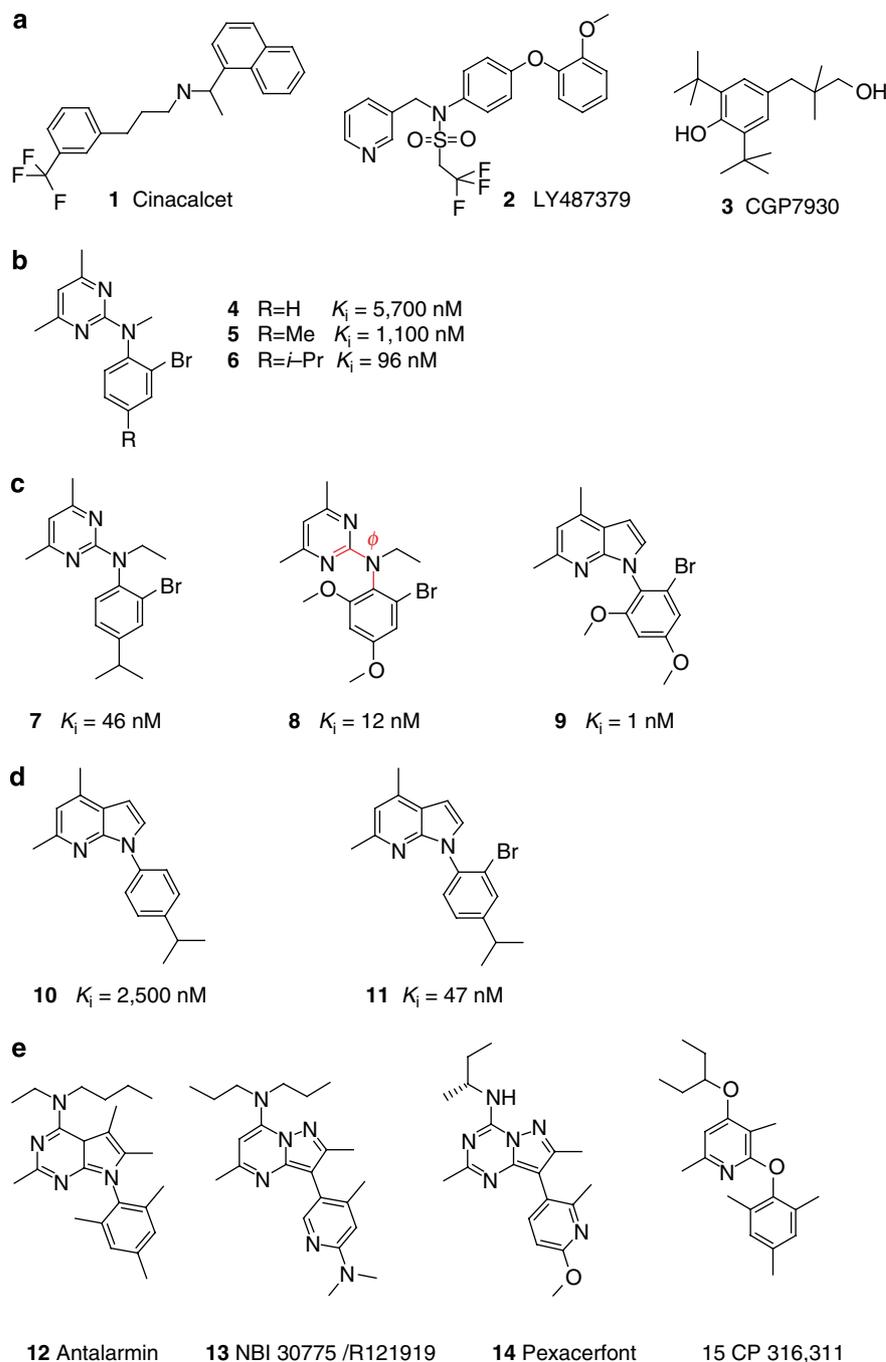
potential value as novel targets for the treatment of multiple diseases. Beyond the simple structural and functional organization of monoamine class A GPCRs, these receptors presented a considerable challenge for small molecule drug development. Allosteric modulation has proven to be a viable and tractable route to the development of small molecule compounds for these receptors, some of which are currently progressing through clinical evaluation.

### Ligand Structure: Drug Development for Peptide GPCRs

To develop a drug for a target of interest, it is first necessary to have in hand a chemical starting point. In the infancy of medicinal chemistry, endogenous organic compounds such as the neurotransmitters norepinephrine and histamine provided the initial chemical starting points. With the explosion of potential new targets to treat CNS diseases came the problem of identifying novel chemical starting points—many of the interesting GPCR targets were activated by endogenous peptide neuromodulators. Peptides themselves are generally considered poor chemical starting points for CNS diseases because they do not readily cross the blood–brain barrier. For drug discovery in general, peptides present challenges in terms of route of administration (usually injection), stability in the body and

ease of synthesis and large-scale production. Consequently new methods were required to identify chemical starting points for the large majority of peptide-activated GPCRs. HTS has been an invaluable technological advancement in identifying chemical starting points for peptide GPCRs. Here we review the scientific processes involved in successfully applying HTS and in developing lead compounds from initial screening hits.

The first example of a successful HTS application illustrates how an initial weak hit was optimized using medicinal chemistry to yield valuable drug-like small molecules. As introduced above, blocking the CRF<sub>1</sub> receptor is a potentially attractive novel mechanism for treating anxiety, depression and other stress-related disorders. However, the peptide CRF itself is not a viable starting point for medicinal chemistry because of its size (41 amino acids) and because its bioactive conformation (an extended  $\alpha$ -helix (Grace *et al*, 2007; Mesleh *et al*, 2007)) is not easily amenable to structural mimicry with a small molecule. In addition the target receptors for depression and anxiety-related disorders are localized in the CNS, thus requiring a brain-penetrating pharmacological agent that will inevitably be used in a chronic manner. This precludes the use of many inconvenient routes of administration such as injectables, suppositories, or inhalants, requiring an orally bioavailable drug. Such requirements and constraints can



**Figure 4.** Small molecule modulators for class B and class C GPCRs. (a) Chemical structure of allosteric modulators of class C GPCRs. (b–e) Lead optimization for CRF receptor antagonists. (b) Lower aromatic substitutions in early CRF<sub>1</sub> receptor lead exploration (Arvanitis *et al*, 1999). (c) Ethyl substituent on central nitrogen and dihedral angle exploration in early lead exploration (Arvanitis *et al*, 1999). The position of the dihedral angle measured for compound **8** is indicated with the red-colored bonds (Hodge *et al*, 1999). This angle is constrained by the bicyclic core in compound **9**. (d) Two-position substituent on lower aromatic required for high affinity binding, suggesting the lower aromatic is held orthogonal to the plane of the core in the bioactive conformation (Hodge *et al*, 1999). (e) Lead CRF<sub>1</sub> receptor antagonists: **12**—Antalarmin (Webster *et al*, 1996); **13**—NBI 30775/R121919 (Chen *et al*, 2004b); **14**—Pexacerfont; **15**—CP-316,311 (Binneman *et al*, 2008).

be satisfied with a small molecule compound, but this development path requires a viable chemical starting point. In the case of the CRF<sub>1</sub> receptor, HTS was used to identify such a starting point.

An initial hit on the CRF<sub>1</sub> receptor was reported in 1999 (compound **4**, Figure 4b), identified from screening the DuPont chemical library in competition against <sup>125</sup>I-CRF

binding to the endogenous CRF<sub>1</sub> receptor in rat brain homogenates (Arvanitis *et al*, 1999). (First detailed report of small molecule development for the CRF<sub>1</sub> receptor, from screening hit to lead molecules.) This compound weakly bound the receptor ( $K_i$  of 5.7 μM) and was shown to act as an antagonist, blocking CRF-stimulated signaling in the rat brain tissue (IC<sub>50</sub> of 20 μM). After identifying this initial hit,

the next step down this drug development path was optimizing the hit into a *bona fide* lead. The first efforts focused on improving the affinity, to maximize the chances of showing efficacy at reasonable levels of compound exposure in experimental animals. The introduction of a simple methyl group at the four-position of the phenyl ring (compound 5, Figure 4b) improved the affinity approximately fivefold ( $K_i$  of 1.1  $\mu$ M). Increasing the size of the 4-methyl group led to the 4-*i*-propyl analog 6 (Figure 4b) with an additional 11-fold improvement in CRF<sub>1</sub> receptor-binding affinity ( $K_i$  of 96 nM). The introduction of a single additional methylene unit (which resulted in a simple ethyl substituent on the linking nitrogen atom at the two-position of the pyrimidine) modestly increased the affinity further (7,  $K_i$  of 46 nM, Figure 4c). Finally, modifying the two- and four-position substituents on the phenyl ring yielded high affinity antagonists exemplified by compound 8 ( $K_i$  of 12 nM, Figure 4c). Nuclear magnetic resonance and X-ray analysis of this monocyclic antagonist had shown that the dihedral angle  $\phi$  between the central core nitrogen and the pyrimidine group was almost equal to 0° (compound 8, Figure 4c; Hodge *et al*, 1999). Subsequently it was demonstrated that conformationally locking the compound to maintain this dihedral angle with the use of a bicyclic core yielded very potent molecules (compound 9,  $K_i$  of 1 nM, Figure 4c; Hodge *et al*, 1999).

The relative orientation of the phenyl group and the planar core heterocycle was also examined. Potentially free rotation of the phenyl group, as in compound 10, (Figure 4d), resulted in low affinity for the CRF<sub>1</sub> receptor ( $K_i$  of 2.5  $\mu$ M, (Hodge *et al*, 1999). Two-position substituents likely impose steric constraints that result in the phenyl ring lying orthogonal to the plane of the core (Kehne and De Lombaert, 2002). This is likely the preferred configuration for bioactivity because the two-position substituents dramatically increase CRF<sub>1</sub> receptor affinity (compound 11,  $K_i$  of 47 nM, Figure 4d). The final area of SAR exploration has been the 'top' alkyl group, a simple methyl in the initial lead optimization efforts, expanded to lipophilic alkyl chains in early lead optimization (eg antalarmin, compound 12, Figure 4e). Similar chemotypes were also reported at this time, including antalarmin and CP-154,526 (Chen *et al*, 1996; Schulz *et al*, 1996). After optimizing for affinity, further drug development efforts have focused on improving the physicochemical characteristics of the molecules themselves to improve pharmacokinetics, typically by reducing lipophilicity using heteroatoms, potentially also increasing basicity (reviewed in Tellew and Luo 2008). These efforts yielded compounds that have entered clinical trials, such as NBI 30775/R121919 (compound 13, Figure 4e; Chen *et al*, 2004b), Pexacerfont (compound 14, Figure 4e) and CP-316311 (compound 15, Figure 4e). Although Pfizer has just recently published that CP-316,311 failed in a major depression clinical trial (Binneman *et al*, 2008), Pexacerfont from Bristol-Myers Squibb has currently completed three independent phase II/

III clinical trials for the potential treatment of generalized anxiety disorder (Clinicaltrials.gov identifier number NCT00481325), major depressive disorder (Clinicaltrials.gov identifier number NCT00135421), and irritable bowel syndrome (Clinicaltrials.gov identifier number NCT00399438). In addition, GlaxoSmithKline (GSK) has recently reported that 876008, a selective CRF<sub>1</sub> receptor antagonist, failed to reach statistically significant differences in the key efficacy endpoints between 876008 and placebo at 12 weeks in a Phase II clinical trial for social anxiety disorder (Clinical trials.gov identifier number NCT00397722). However, a second selective CRF<sub>1</sub> receptor antagonist (561679) has very recently begun a Phase II clinical trial in major depressive disorder (Clinical trials.gov identifier number NCT00733980).

In the modern era HTS remains to an extent an exercise in serendipity, inasmuch as success requires that the chemical space of the library being screened intersects with that of an active chemical structure on the target—in essence the union of the drugability of the target and the drug-likeness of the small molecule. Indeed the identification of the initial lead for the CRF<sub>1</sub> receptor was almost entirely serendipitous. For other GPCRs the chance element has been reduced as much as possible by biasing the chemical space being screened using *a priori* assumptions regarding the types of chemical structures that are likely to interact with the target in the required manner. The structure of the receptor and the ligand has proved useful in this process. For example, activation of melanocortin 4 (MC4) receptors in the hypothalamus has been proposed as a potential means to exert central control over energy balance and food intake in treating obesity and other metabolic disorders. The amino-acid sequence of the MC4 receptor shares considerable homology with that of other GPCRs for which drugs had already been identified, such as the ghrelin receptor, which binds growth-hormone secretagogue compounds (Bondensgaard *et al*, 2004). A directed screen of a library containing growth-hormone secretagogue compounds yielded a moderate potency (110 nM) MC4 receptor hit (Sebhat *et al*, 2002). Compounds elaborated from this initial lead are similar to the bioactive structure of the endogenous peptide agonist  $\alpha$ -melanocyte-stimulating hormone and synthetic peptide analogues (Sebhat *et al*, 2002; Sun *et al*, 2004). This information was used to guide further medicinal chemistry efforts to optimize small molecule potency for the MC4 receptor. Additional optimization provided selectivity for the MC4 receptor over the other four MC receptor subtypes, and the appropriate physicochemical profile to allow *in vivo* efficacy in disease models to be demonstrated (reviewed in Chen *et al*, 2004a; Todorovic and Haskell-Luevano, 2005).

It is important to bear in mind that the level of understanding of how these molecules function is still being enhanced. There are many examples of drug discovery efforts that have failed despite the 'similarity' of the target GPCRs to others in the same family. These targets

at this time are apparently not drugable but clearly, with technologies evolving, further elucidation of the mechanisms involved, specific functional conformations and possible auxiliary protein requirements all may offer insights into our abilities to identify small molecules that have the potential of becoming novel therapeutics for diseases that can be affected by these targets.

## MEMBRANE-BOUND ION CHANNELS

### Drug Development Challenges in Targeting Modulation of Ion Channel Function

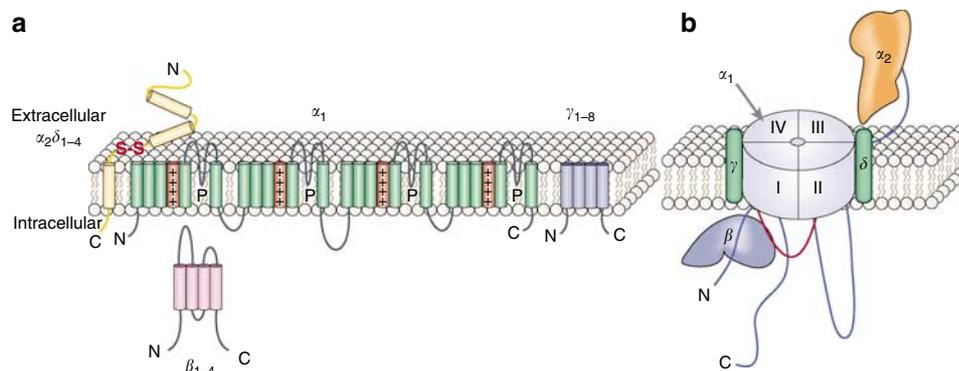
Ion channels represent a diverse group of integral membrane proteins that regulate the passage of ions across cell membranes. They are ubiquitously expressed throughout the body and critically involved in basic physiological processes such as regulation of membrane potential, signal transduction, secretory function, and cellular plasticity. These features, on the one hand, make them compelling drug targets and on the other, limit their drugability. This is particularly true when aiming to develop orally bioavailable CNS therapeutics, as essential peripheral processes are also highly regulated by ion channel function.

Classification of ion channels can be made using various methods. Although ion channels are specific for certain ions (sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), calcium ( $\text{Ca}^{2+}$ ), chloride ( $\text{Cl}^-$ )) or for classes of ions (cations or anions), they have most successfully been organized by how they are regulated. Ion channels transition between open and closed states by a complex process called 'gating', whereby a conformational change takes place allowing for or blocking the passage of ions through the channel pore. If the probability a channel will be in a particular state is regulated by membrane potential, the channel is termed 'voltage-gated'. Nearly all cells have some voltage-sensitive ionic permeability and these channels can be further classified by the kinetics of their gating. In addition to the complex processes by which

channels gate, their structural organization provides another level of complexity when pursuing the development of drug-like molecules. If the probability a channel will be in a particular state is regulated by a chemical agent (ligand), the channel is termed 'ligand-gated'. Like GPCRs, ligand-gated channels rely on the binding of a ligand to a specific receptor, although in this case the receptor is specifically located on the channel, and this interaction induces the conformational change necessary to open or close the channel. Ligand-gated channels often contain binding sites for multiple ligands that can each differentially regulate channel function either alone or in concert with other ligands. In this section, it will be shown how an understanding of the structural components, as well as the gating mechanisms regulating ion channel function has been applied to successfully develop drug-like molecules for ion channels.

### Voltage-Gated Ion Channels: Novel Approaches to Modulation of $\text{Ca}_v2.2$ in Drug Development

Most voltage-gated calcium channels are multisubunit complexes (Catterall, 2000; Yu *et al*, 2005; Zhorov and Tikhonov, 2004; Figure 5), containing a principal pore-forming subunit ( $\alpha_1$ ), which determines the permeation properties of the channel. This subunit also contains the binding site for most commercially available pharmaceuticals known to interact with calcium channels, as well as the voltage sensor, which regulates gating. The ten  $\alpha_1$  subunits are divided into three families based on the voltage required for their activation (Bean, 1989). L-type calcium channels ( $\text{Ca}_v1.1-1.4$ ), signified by  $\alpha_{1C}$ ,  $\alpha_{1D}$ ,  $\alpha_{1F}$ ,  $\alpha_{1S}$ , P- and Q-type calcium channels ( $\text{Ca}_v2.1$ ), signified by  $\alpha_{1A}$ , N-type calcium channels ( $\text{Ca}_v2.2$ ), signified by  $\alpha_{1B}$ , and R-type calcium channels ( $\text{Ca}_v2.3$ ), signified by  $\alpha_{1E}$ , are all activated by high voltage (potentials greater than  $-40$  mV). T-type calcium channels ( $\text{Ca}_v3.1-3.3$ ), signified by  $\alpha_{1G}$ ,  $\alpha_{1H}$ ,  $\alpha_{1I}$ , are activated by low voltage (more negative potentials). The  $\alpha_1$



**Figure 5.** Schematic of a voltage-gated calcium channel. (a) Proposed membrane topology and structural domains of calcium channel subunits. Four homologous domains (I–IV), each with six transmembrane segments, comprise the pore-forming  $\alpha_1$  subunit. The extracellular  $\alpha_2$  subunit is disulfide linked to the transmembrane  $\delta$ -subunit, although these subunits arise from a single gene product that is posttranslationally cleaved. The  $\beta$ -subunit resides intracellularly and interacts with the loop connecting domain I to II. The  $\gamma$ -subunit has four transmembrane segments. (b) Vertical cross section showing the quadrameric arrangement of homologous domains to create the pore-forming  $\alpha_1$  subunit, the central calcium permeable channel and its proposed relationship to the auxiliary subunits,  $\alpha_2\delta$  and  $\beta$ .

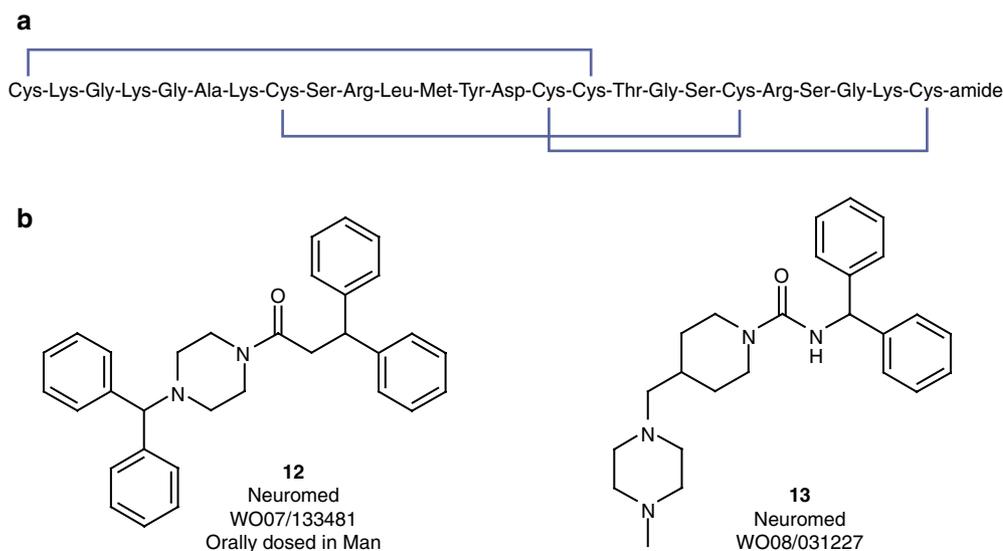
subunit has four homologous domains (I–IV), each comprised six  $\alpha$ -helical TM spanning segments (S1–S6), with a membrane-reentrant loop (P-loop) between S5 and S6 (Yang *et al*, 1993). This region (S5, S6, P-loop) comprises the ion-conducting pore and the selectivity filter. A combination of biophysical, mutagenesis and structural studies of the voltage-gated sodium and potassium channels has provided evidence for localization of the voltage-sensor regions on S4, which contains a pattern of positively charged amino acids critical to the gating mechanism (Bezanilla, 2002). The majority of therapeutically relevant ion channel drugs bind to the  $\alpha_1$  subunit and inhibit ion flow, either by blocking the pore directly or by interfering with the gating mechanism. Fewer marketed drugs exist, which act to enhance ion flow through the channel by interacting with this subunit.

In addition to the principal pore-forming subunit ( $\alpha_1$ ), voltage-gated calcium channels are associated with auxiliary subunits (Figure 5) that modify their expression, functional properties, and subcellular localization. These include  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  (Arikath and Campbell, 2003).  $\alpha_2$  and  $\delta$  are encoded by the same gene, which is posttranslationally cleaved to form the subunit  $\alpha_2\delta$ , of which there are four types, exhibiting tissue-specific localization.  $\alpha_2$ , localized extracellularly and involved in regulation of  $\alpha_1$  function, is disulfide linked to  $\delta$ , which has a TM segment involved in anchoring the subunit to the membrane (Wiser *et al*, 1996). There are also four  $\beta$ -subunits, comprised four  $\alpha$ -helical segments that are localized intracellularly and regulate cell surface expression, as well as modulation of gating. They are involved in such functions as causing enhanced activation upon depolarization and altered rate and voltage dependence of inactivation. The  $\beta$ -subunits interact with the intracellular loop between domains I and II of the  $\alpha_1$  subunit (Dolphin, 2003). There are eight  $\gamma$ -subunits, each containing four TM segments, which are involved in

assembly and cell surface expression of not only voltage-gated calcium channels, but potentially other receptors and membrane signaling proteins (Kang *et al*, 2001). There are only a few examples of therapeutically relevant ion channel drugs that bind to auxiliary subunits, however this approach has more recently gained increasing attention with the clinical and commercial success of gabapentin (Neurontin<sup>®</sup>) and pregabalin (Lyrica<sup>®</sup>).

N-type calcium channels ( $\text{Ca}_v2.2$ ) have been of particular interest in CNS drug discovery because of their almost exclusive localization to neurons, most particularly to presynaptic terminals, where they are involved in regulating the release of neurotransmitters by allowing an influx of calcium necessary for exocytotic vesicle fusion (Bertolino and Llinas, 1992; Dunlap *et al*, 1995). The discovery that these channels are also involved in transmission of nociceptive information in the spinal cord, coupled with the increase in expression of both the  $\alpha_{1B}$  and  $\alpha_2\delta$  subunits in dorsal root ganglion neurons following nerve injury, led to an increased interest in targeting this channel for the development of pain therapies (Cizkova *et al*, 2002; Heinke *et al*, 2004; Luo *et al*, 2002; Westenbroek *et al*, 1998). In addition, 20–30 amino-acid peptide  $\omega$ -conotoxins from marine cone snails (GVIA from *Conus geographus*, MVIIA, from *Conus magus*, CVID from *Conus catus*; Ellinor *et al*, 1994; Olivera *et al*, 1994) have been shown to bind with high affinity (nM) and selectivity to  $\text{Ca}_v2.2$  and reduce measures of neurotransmission and pain response in behavioral models of nerve injury and inflammation, when administered into the spinal cord. The toxins bind to the  $\alpha_{1B}$  subunit in the P-loop region of the channel pore and potently block the channel directly (Feng *et al*, 2003).

A critical advance was made when Elan Pharmaceuticals developed a synthetic version of  $\omega$ -conotoxin MVIIA, referred to as ziconotide or SNX-111 (see Figure 6a for peptide sequence and refer to <http://www.3dchem.com/>



**Figure 6.** (a) Peptide sequence of ziconotide (also refer to <http://www.3dchem.com/molecules.asp?ID=260> for the 3-D structure). (b) Examples of  $\text{Ca}_v2.2$  antagonists from Neuromed.

molecules.asp?ID=260 for the 3-D structure of this peptide), which similarly binds  $\alpha_{1B}$  and blocks the channel directly (Bowersox *et al*, 1996; Wang *et al*, 2000). Ziconotide was the first selective N-type calcium channel blocker to be tested in clinical trials and has recently been approved for the treatment of severe chronic pain associated with cancer, AIDS and neuropathies, under the trade name Prialt<sup>®</sup> (Atanassoff *et al*, 2000; Brose *et al*, 1997; McGivern, 2007; Miljanich, 2004; Staats *et al*, 2004). Although Prialt does not induce tolerance and shows efficacy in patients refractory to opioid therapies, it is not without limitations. In addition to requiring intrathecal administration, Prialt induces significant orthostatic hypotension, most likely because of interaction with  $Ca_v2.2$  on neurons regulating blood pressure. Systemic administration inhibits sympathetic control of multiple cardiovascular functions regulated by peripheral  $Ca_v2.2$  (Penn and Paice, 2000).

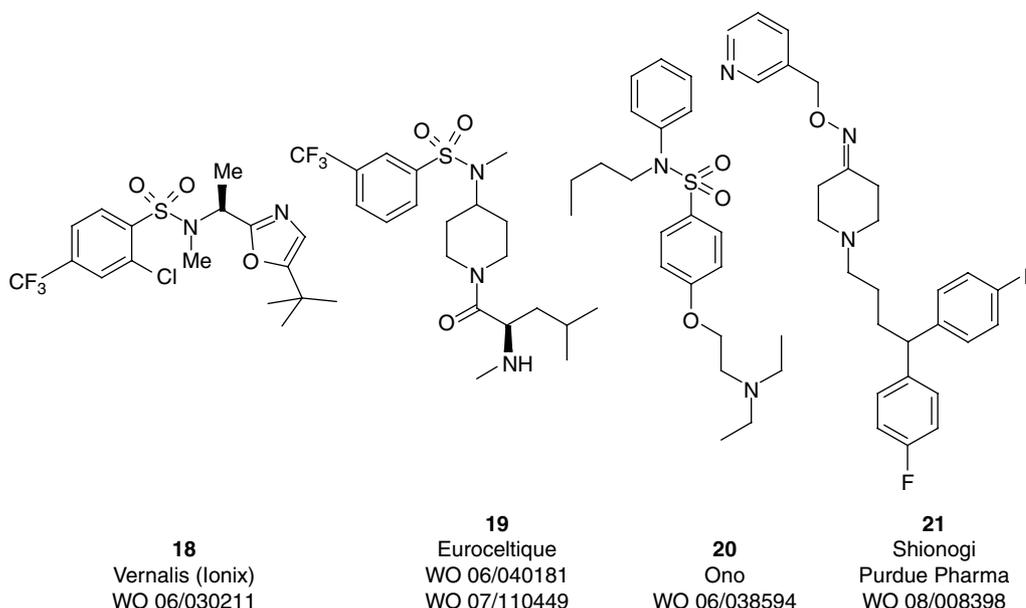
Despite the known limitations of Prialt, its potent efficacy as a pain therapeutic has compelled many companies to engage in the development of small molecule antagonists of  $Ca_v2.2$  (Birch *et al*, 2004; Markman and Dworkin, 2006; McGivern, 2006; Sabido-David *et al*, 2004; Schroeder *et al*, 2006; Yogeewari *et al*, 2007). The hope is that a small molecule would be orally bioavailable, be selective *versus* other channels and have an improved safety profile over Prialt. To date only one company, Neuromed, has successfully advanced a small molecule N-type calcium channel blocker into clinical trials (NMED-160). Like ziconotide and the  $\omega$ -conotoxins, NMED-160 binds to the  $\alpha_{1B}$  subunit. However, unlike these peptides, it preferentially binds to the subunit in a frequency- or use-dependent manner (Snutch, 2003). Use dependence is a phenomenon whereby a higher frequency of action potentials results in a higher degree of channel block. Inhibition increases as channels are 'used' (for reviews of potential mechanisms by which inhibitors can take advantage of changes in gating and permeation processes, see Birch *et al*, 2004; Winquist *et al*, 2005). *In vivo* electrophysiological recordings have demonstrated an increased frequency of firing in nociceptive neurons of animals experiencing neuropathic pain. It is thought that targeting channels in this state would provide a degree of selectivity that would potentially block  $Ca_v2.2$  channels involved in mediation of pain responses whereas sparing the role these channels play in normal central and peripheral sympathetic functions.

NMED-160 is a potent use-dependent blocker of  $Ca_v2.2$ . Electrophysiological patch-clamp data in HEK cells expressing  $Ca_v2.2$  showed a more potent block of channel function at higher frequencies of stimulation (IC<sub>50</sub> of 120 nM at 0.03 Hz compared to 50 nM at 0.2 Hz), with some selectivity over P- and Q-type calcium channels (1.6-fold) and L-type calcium channels (8-fold). NMED-160 has been shown to be orally efficacious in animal models of neuropathic pain and to be without serious side effects, including effects on cardiovascular end points, in phase I clinical trials. Although the chemical structure of NMED-160 has not been publically disclosed, a recently published Neuromed

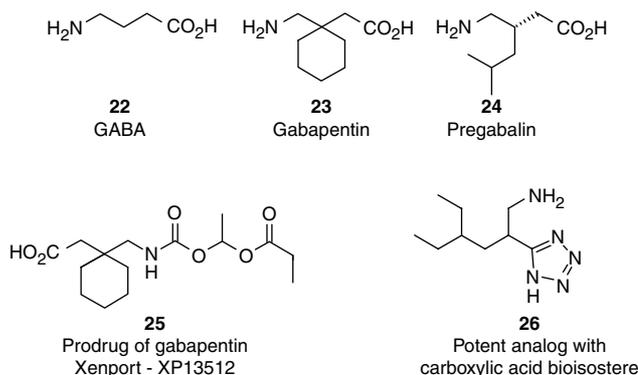
patent (WO 2007133481; Snutch and Fisher, 2007) discloses the structure of compound 16 (Figure 6b) for which human bioavailability data is presented. Merck & Co, under license from Neuromed, was developing NMED-160 for the potential treatment of chronic pain. By March 2006, the drug was in phase II trials, however, as defined in their press release on August 8th, 2007, Neuromed and Merck discontinued development of this drug stating 'that MK-6721 (also known as NMED-160) does not demonstrate the ideal pharmaceutical characteristics considered necessary to advance the compound further in development.' (please refer to the original press release for details at <http://www.neuromed.com/news/pressr.php?nr=7&txt=&pyear=2007>). The collaboration between Merck and Neuromed has continued toward identifying a compound with improved characteristics and several new patents have emerged (eg see compound 17, Figure 6b; Pajouhesh *et al*, 2008). A number of other companies are also active in this area (see compounds 18–21, Figure 7), although to date, no compounds from these efforts have yet been advanced into clinical trials.

As mentioned above, the majority of ion channel therapeutics have interactions with the pore-forming subunit,  $\alpha_1$ . In the case of  $Ca_v2.2$ , this approach has been successful for ziconotide and progress has been made with newer compounds targeting a more specialized interaction with this subunit, use-dependent blockade. A much smaller number of ion channel therapeutics have been developed, which target an auxiliary subunit. The most compelling of these interact with the  $\alpha_2\delta$  subunit of  $Ca_v2.2$  and are marketed therapies for the treatment of multiple CNS indications.

Gabapentin, 1-(aminomethyl)cyclohexaneacetic acid (Neurontin, Pfizer), was originally developed as a chemical analogue of GABA (compounds 22 and 23, Figure 8) to reduce the spinal reflex for the treatment of spasticity and was found to have anticonvulsant activity in various animal models of seizure (Taylor *et al*, 1998). In addition, it also displays efficacy in a number of animal models of inflammatory and neuropathic pain (Hunter *et al*, 1997; Kaneko *et al*, 2000; Shimoyama *et al*, 1997). Clinically, gabapentin is indicated as an add-on medication for the treatment of partial seizures and a variety of neuropathic pain syndromes, such as postherpetic neuralgia (Curran and Wagstaff, 2003; Plaghki *et al*, 2004). It is also claimed to have shown benefit in other clinical disorders such as fibromyalgia, anxiety, bipolar disorder, and hot flashes, although it has not been approved for these indications (Arnold *et al*, 2007; Cheng and Chiou, 2006). The possible mechanisms of action for gabapentin have been extensively studied and several hypotheses have been postulated (Cheng and Chiou, 2006; Taylor *et al*, 1998). Originally, it was thought that effects of gabapentin were mediated by its interaction with GABA receptors, however, despite early reports of an interaction with GABA<sub>B</sub> receptors in recombinant cell lines, *Xenopus oocytes* and rat brain slices, later imaging and pharmacological evaluations did not



**Figure 7.** Examples of  $\text{Ca}_v2.2$  antagonists from the patent literature (Benjamin *et al*, 2006; Chen *et al*, 2007; Hobbs *et al*, 2006; Matsumura *et al*, 2008; Ohtani *et al*, 2006).



**Figure 8.** GABA, gabapentin, and analogs.

support an interaction with either receptors or transporters of the GABA system (Bertrand *et al*, 2001; Cheng *et al*, 2004; Shimizu *et al*, 2004; Taylor *et al*, 2007).

A great deal of work was undertaken to isolate, validate, and characterize the binding site for gabapentin as the  $\alpha_2\delta$  subunit of  $\text{Ca}_v2.2$ . There are four variants of the  $\alpha_2\delta$  subunit and gabapentin binds with high affinity to  $\alpha_2\delta$ -1 and  $\alpha_2\delta$ -2, which vary slightly in their distribution in the CNS (Wiser *et al*, 1996). For a number of years, the mechanism of how binding to this subunit resulted in analgesic effects through  $\text{Ca}_v2.2$  was elusive. Early reports indicated an inhibitory effect of gabapentin on calcium currents (Bayer *et al*, 2004; Rock *et al*, 1993; Sutton *et al*, 2002), but many subsequent studies were unable to replicate this work, perhaps because of the varied experimental conditions between laboratories, such as the subunit composition of the native calcium channels in different preparations. It is thought that the role

of the  $\alpha_2\delta$  subunit is in regulating the membrane insertion of the  $\alpha_1$  subunit, as well as in modulating the voltage dependence and kinetic properties of channel gating. Very recently it has been shown that chronic application of gabapentin reduces plasma membrane expression of calcium channels in heterologous expression systems, suggesting that gabapentin, through its interaction with the  $\alpha_2\delta$  subunit, causes a disruption of calcium channel trafficking (Hendrich *et al*, 2008).

Despite its widespread use in the clinic and a compelling safety profile (somnolence and dizziness most prevalently noted), gabapentin must be titrated to quite high levels (g/day) to achieve efficacy and many patients experience suboptimal benefit, or in the case of pain management, little pain relief at all. However, following quickly on the heels of gabapentin was the approval of pregabalin (Lyrica), also brought to the clinic by Pfizer. Pregabalin (compound 24, Figure 8) has a sixfold higher binding affinity for  $\alpha_2\delta$  than gabapentin and has been shown to have greater bioavailability (Gajraj, 2007; Taylor *et al*, 2007). In addition, pregabalin has been shown to reduce calcium influx at a number of nerve terminals, resulting in a decrease in the release of such neurotransmitters as glutamate, norepinephrine and substance P, providing another possible means by which it exerts its effects clinically (Fehrenbacher *et al*, 2003). Pregabalin is currently approved in the United States as an adjunctive treatment for partial seizures and for the treatment of pain syndromes such as diabetic neuropathy, postherpetic neuralgia, and fibromyalgia, making it the first drug approved for more than one neuropathic pain condition (Crofford *et al*, 2005; Dworkin *et al*, 2003; Lesser *et al*, 2004; Rosenstock *et al*, 2004).

Additional indications have been approved in Europe, including the use of pregabalin in anxiety disorders (Sabatowski *et al*, 2004).

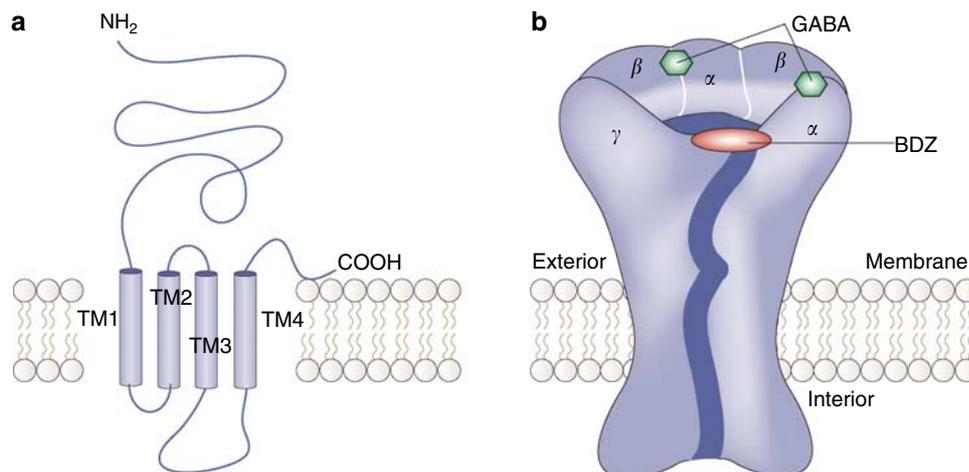
Although many groups have worked towards the development of more efficacious analogues of gabapentin, in general these efforts have been plagued by the poor bioavailability of simple amino acid based derivatives. Pregabalin not only binds  $\alpha_2\delta$ , but is also a substrate for active transport, believed to enter into the CNS by the system L amino-acid transporter (Su *et al*, 1995, 2005). To improve bioavailability without relying on an active transport mechanism, acid bioisosteres (a substituent or group that imparts similar biological properties to a chemical compound, but enhances the physical properties for drug development) and prodrugs have been employed (Field *et al*, 2007). (Comprehensive review of the development of drug-like ligands for  $\alpha_2\delta$ .) The prodrug XP13512 (compound 25, Figure 8) has been shown to improve exposure in two different species compared to dosing gabapentin itself. XP13512 has been demonstrated to increase plasma levels of gabapentin by 9-fold in the monkey and by 17-fold in the rat following oral administration, and 34-fold in the monkey following direct intracolonic administration compared to gabapentin by the same route (Cundy *et al*, 2004). Compound 26 (Figure 8) is an example of a compound with a tetrazole as a carboxylic acid bioisostere, which was shown to have potent binding to  $\alpha_2\delta$  and demonstrated robust anticonvulsant activity *in vivo*. Although there is still a great deal to understand about the mechanisms within which an interaction with the  $\alpha_2\delta$  subunit can alter calcium channel function, the targeting of auxiliary subunits is a compelling area to explore for the development of novel therapeutics.

## Ligand-Gated Ion Channels: Subtype-Selective Modulation of GABA<sub>A</sub> in Drug Development

GABA is the major inhibitory neurotransmitter in the mammalian CNS and mediates fast inhibitory synaptic interactions by the GABA<sub>A</sub> receptor. Binding of GABA to GABA<sub>A</sub>, localized on postsynaptic membranes, results in a conformational change in the ion channel, allowing the flow of chloride ions into the cell. This causes hyperpolarization of the membrane potential and inhibition of neuronal activity.

The GABA<sub>A</sub> receptor belongs to a family of pentameric ligand-gated ion channels that also includes nicotinic acetylcholine, strychnine-sensitive glycine, and serotonin 3 (5HT<sub>3</sub>) receptors (Le Novère and Changeux, 2001). Sixteen different subunits can be components of the GABA<sub>A</sub> channel,  $\alpha$ (1–6),  $\beta$ (1–3),  $\gamma$ (1–3),  $\delta$ ,  $\epsilon$ ,  $\pi$ , and  $\theta$  (Barnard *et al*, 1998; Whiting, 2003). Five subunits are arranged around a central pore that makes up the actual ion channel (Figure 9). Each subunit has a large extracellular N-terminal domain, which incorporates part of the agonist-/antagonist-binding site, followed by three membrane-spanning domains (TM1–TM3), an intracellular loop of variable length and a fourth membrane-spanning domain (TM4) with the C-terminal end being extracellular. The second membrane-spanning domain (TM2) forms the wall of the channel pore. GABA<sub>A</sub> receptors are generally comprised two  $\alpha$ -, two  $\beta$ - and one  $\gamma$ -subunit (McKernan and Whiting, 1996; Whiting, 2003).

The GABA<sub>A</sub> receptor contains distinct-binding sites for many pharmacologically active compounds, including anesthetics, anticonvulsants, anxiolytics, and sedative-hypnotics. GABA binds to the extracellular N-terminal domain of each  $\alpha$ -subunit, near the interface between the  $\alpha$ - and



**Figure 9.** Schematic of a GABA<sub>A</sub> receptor. (a) Proposed membrane topology and structural domains of a GABA<sub>A</sub> receptor subunit. Each subunit contains a large extracellular agonist-/antagonist-binding N-terminal domain, four transmembrane domains and a large cytoplasmic loop between M3 and M4. The second membrane-spanning domain (M2) forms the wall of the channel pore. (b) Vertical cross section showing the pentameric arrangement of subunits (2 $\alpha$ , 2 $\beta$ , and 1 $\gamma$ ) necessary to create the central chloride permeable channel. The recognition sites for GABA are indicated at the interface between the  $\alpha$ - and  $\beta$ -subunits, and for the benzodiazepines at the interface between  $\alpha$ - and  $\gamma$ -subunits.

$\beta$ -subunits. Most pharmacologically active compounds do not interact directly with the GABA-binding site, but act as allosteric modulators, either reducing or enhancing the actions of GABA at its receptor. These include such drugs as the synthetic steroid anesthetic, alphaxalone, a positive allosteric modulator of GABA activity, which interacts with  $\delta$ -subunit-containing GABA<sub>A</sub> receptors, and the loop diuretic, furosemide, which interacts with  $\alpha_6$  subunit-containing GABA<sub>A</sub> receptors to allosterically decrease GABA activity. In addition to distinct-binding sites for neuroactive steroids and furosemide, GABA<sub>A</sub> receptors contain binding sites for picrotoxin, sedative-hypnotic barbiturates, ethanol, inhalation anesthetics, and a number of divalent cations. The most extensively studied of the allosteric modulators of GABA on GABA<sub>A</sub> receptors are the benzodiazepines (Johnston, 1996), typified by the compounds diazepam (Valium<sup>®</sup>) and alprazolam (Xanax<sup>®</sup>; compounds 27 and 28, Figure 10), and widely prescribed for the treatment of anxiety disorders for many decades.

Benzodiazepines bind the GABA<sub>A</sub> receptor at a site situated at the interface between the  $\alpha$ - and  $\gamma$ -subunits (Benson *et al*, 1998; Minier and Sigel, 2004). Although benzodiazepines are highly efficacious compounds, their

use is limited by significant side effects, which can include sedation, amnesia, tolerance, dependence, and withdrawal (Buffett-Jerrott and Stewart, 2002; Shader and Greenblatt, 1993; Woods, 1998). These effects are believed to be mediated by their specific interactions with the GABA<sub>A</sub> receptor. Benzodiazepines have been shown to bind to and potentiate GABA activity at GABA<sub>A</sub> receptors containing  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , or  $\alpha_5$  combined with  $\beta\gamma$ -subunits, but not those combinations containing  $\alpha_4$  or  $\alpha_6$  (Benson *et al*, 1998; Rudolph *et al*, 1999). A specific point mutation in the  $\alpha$ -subunits has been identified as conferring this selectivity and several transgenic mouse lines have been generated to further explore the role of these subunits in the actions of benzodiazepines. Studies utilizing these transgenic mice have provided evidence for the role of receptors containing the  $\alpha_1$  subunit in mediating the sedative effects of diazepam, receptors containing the  $\alpha_2$  subunit in mediating the anxiolytic effects of diazepam and receptors containing the  $\alpha_5$  subunit in mediating both the effects of diazepam on memory functions and on tolerance to the sedative effects of the drug (Collinson *et al*, 2002; Low *et al*, 2000; McKernan *et al*, 2000; Reynolds *et al*, 2001).

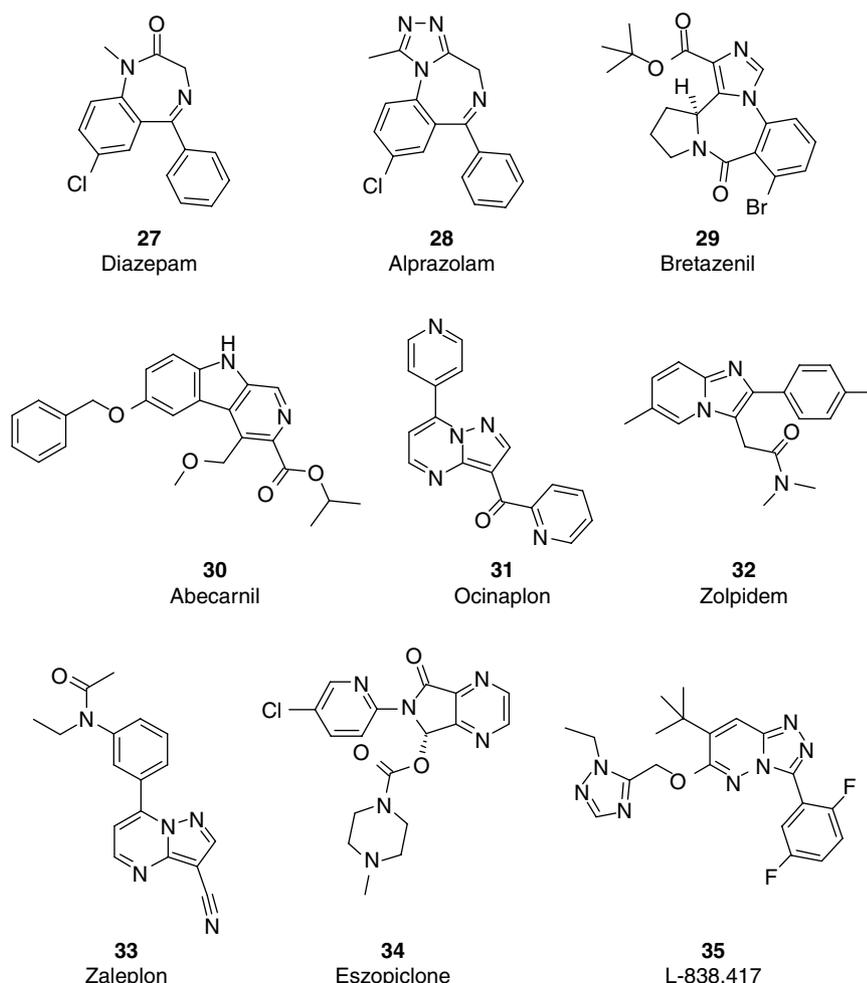


Figure 10. Examples of GABA<sub>A</sub> modulators.

With this knowledge in hand, drug discovery efforts aimed at providing a new and improved benzodiazepine have taken a number of paths. Some efforts have attempted to develop nonselective, partial agonists of the GABA<sub>A</sub> receptor, with the goal of producing efficacy with reduced side effect liability. Several compounds have progressed to the clinic, but whereas they have demonstrated anxiolytic efficacy in animal models with reduced effects on sedation, muscle relaxation, and withdrawal, these effects were not completely borne out in man, and these attempts were discontinued (Figure 10). Bretazenil (Roche; compound 29, Figure 10; Busto *et al*, 1994; van Steveninck *et al*, 1996) demonstrated clinical efficacy but was unable to show a separation between the anxiolytic benefit and the sedative liability. Abecarnil (Schering; compound 30, Figure 10; Dubinsky *et al*, 2002; Stephens *et al*, 1990), having demonstrated partial agonism in some animal models, was unable to demonstrate significant efficacy once in the clinic. Ocinalplon (DOV; compound 31, Figure 10; Lippa *et al*, 2005), was most promising for its benzodiazepine-like efficacy and absence of untoward side effects in man, however, was unfortunately discontinued because of enzyme elevations in liver function tests.

A second approach to a new generation of benzodiazepines has been undertaken with an attempt to synthesize compounds with selective affinity for different GABA<sub>A</sub> receptor subunits. This has proven to be successful in the development of novel therapeutics for the treatment of insomnia. The marketed drugs, zolpidem (Ambien<sup>®</sup>, compound 32, Figure 10), zaleplon (Sonata<sup>®</sup>; compound 33, Figure 10), and eszopiclone (Lunesta<sup>®</sup>; compound 34, Figure 10) all have varying pharmacological profiles with respect to their interactions with GABA<sub>A</sub> subunits, but all of these compounds have a preferred affinity for the  $\alpha_1$  subunit (Petroski *et al*, 2006; Sanna *et al*, 2002). Although these drugs have proven to be effective in preferentially targeting sedation by selective interaction with  $\alpha_1$ , when present in high concentrations, they retain the potential liabilities associated with the nonsubunit-selective classical benzodiazepines such as triazolam (Halcion<sup>®</sup>). Similar attempts have been made to develop  $\alpha_2$ - or  $\alpha_{2/3}$ -selective compounds for the treatment of anxiety. L-838,417 (Merck; compound 35, Figure 10; McKernan *et al*, 2000), a positive modulator of  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$ , but a neutralizing modulator at  $\alpha_1$  (no effect on GABA activity by  $\alpha_1$  but able to block activity of allosteric modulators at this site), showed promising effects preclinically but was never seen to enter the clinic. Numerous other compounds have been described with somewhat selective profiles, but as yet, a clinically efficacious compound has not emerged from these efforts (Da Settimo *et al*, 2007; Johnston, 2005).

Given the complexity of the interaction of classical benzodiazepines with multiple subunit combinations of the GABA<sub>A</sub> receptor, it is not surprising that drug discovery efforts to find subtype-selective compounds has been undertaken, nor that the process to bring these efforts to fruition is a difficult one. The development of the  $\alpha_1$ -

selective sedative/hypnotics however, provides evidence for the validity of this approach and the impetus for moving forward in these endeavors.

### Drug Development for Ion Channel Modulators: HTS Limitations

A significant barrier to the development of successful ion channel modulators is the difficulty in evaluating the interaction of novel compounds with ion channel targets experimentally and in a manner by which drug discovery efforts can progress in a timely fashion. Traditional high-throughput methods, such as those that have proven successful for GPCR targets have not demonstrated the same promise. Establishment of stable cell lines for use in HTS has been hampered by the need to selectively choose and coexpress the preferred subunit combinations necessary to elicit proper ion channel function while maintaining cell survival. This has often been difficult, particularly with voltage-gated ion channel expression systems. In addition, because of the need to develop subtype-selective compounds, multiple cell lines, expressing ion channels that are closely related to the primary target, must be available for discrete assessment of channel selectivity. Historically, binding assays, whereby novel chemical entities are screened for their ability to displace a radiolabeled ligand specific to the site of interest on the channel have not proven to be reliable in predicting ion channel function (Denyer *et al*, 1998). In addition, by virtue of the novelty of the interactions that are being targeted, specific radioligands have been difficult to develop. High-throughput cell-based assays aimed at evaluating ion channel function have been somewhat more successful but are not without their limitations. The measurement of ion flux through the use of radiotracers is commonly employed in the study of potassium and sodium channels (Mattheakis and Savchenko, 2001; Parihar *et al*, 2003; Xu *et al*, 2001). This methodology provides a direct measure of channel function and a good correlation with electrophysiology but requires a high level of channel expression has poor temporal resolution and is quite costly. The measurement of changes in ion concentration using fluorescent detection methods is common for ions such as calcium where well-established indicators are available (eg Fura-2, Fluo-3, Fluo-4), but development of these assays for other ions has been hindered by the lack of selective and sensitive ion-specific indicators (Wolff *et al*, 2003). In these cases, specific dyes that are sensitive to changes in the membrane potential have been employed to provide an indirect measure of channel function. This methodology has seen the most utility, but delivers the most variable correlation with direct electrophysiological methods. In addition, in the case of voltage-gated channels, the mechanism by which the activation of the channel takes place is artificially induced by a toxin or a drug rather than an electrical impulse and a high degree of false positives/negatives can be anticipated.

The gold standard for assessment of ion channel activity was introduced in the late 1970s by Neher and Sakmann (Hamill *et al*, 1981; Neher and Sakmann, 1976). The whole-cell patch-clamp technique allows the direct recording of ion fluxes across single ion channels or cell membranes and has become the ultimate tool for studying ion channel properties. It provides a detailed set of data on ion channel function with incomparable time resolution (ms) and sensitivity (picoamperes). Unfortunately, the conventional manual patch-clamp technique is time-consuming, low-throughput, and requires highly skilled electrophysiologists, rendering it unsuitable for large-scale high-throughput drug discovery. Recent advances however, have been made in the development of medium throughput patch-clamp systems, allowing for automation of the technique (Farre *et al*, 2007; Wood *et al*, 2004). Some of these systems include PatchXpress™ from Axon Instruments, IonWorks HT™ from Molecular Devices and QPatch™ from Sophion Bioscience. The advent of these technologies holds promise for a path forward for higher throughput ion channel drug discovery for the pharmaceutical industry in the future.

## DRUG DISCOVERY CHALLENGES OF THE FUTURE

There are many hurdles on the road to the discovery and development of potential therapeutics to treat human disease, not the least of which is the fundamental understanding of the target related to a disease state and the drugability of this target, making it amenable to interaction with novel small molecule therapeutics. Serendipity is important in the identification of most drugs on the market today. With the elucidation of the human genome and the promise of selectively specifying molecular targets for various disease states, future efforts are relying more and more on technology-driven initiatives to identify specific molecular interactions that can provide the efficacy required in the disease whereas minimizing potential liabilities. For example, initiatives such as the Molecular Library Screening Center Network, part of the NIH roadmap for Medical Research, provides funding for the discovery of methods that can identify small molecules through novel interactions for which selective small molecule probes are not currently available. These will ideally help in the validation of targets that can play a modulatory or reversal role in the disease. This provides the necessary emphasis on understanding the true drugability of a target and less on the serendipitous findings of currently available random screening processes.

Recent reports expanding the notion of receptor dimerization across different GPCR classes represents a new and increasingly complex dimension to drug discovery. For example, utilizing state-of-the-art techniques the recent description of heterodimer formation between the serotonin 5-HT<sub>2A</sub> (class A) receptor and the mGluR2 (class C) receptor and this dimer is involved in psychosis (Gonza-

lez-Maeso *et al*, 2008) is evidence that ‘drugability’ of targets need not be restricted to single molecular entities with a nice well-defined pocket that can be chemically targeted. In fact multiple drug target opportunities exist in this situation ranging from the classic small molecules interacting within the same pocket as the endogenous ligands for these receptors, through allosteric interactions that target a conformational change in the receptor/protein, to the targeting of the molecular interaction of the dimer itself and the subsequent differential regulation of intracellular signaling mechanisms that this dimer generates (Gonzalez-Maeso *et al*, 2008). Finally, there is a wealth of emerging information from both the biological disciplines (bioinformatics, genomics, proteomics, and other -omics) and the chemistry of small molecule discovery that needs to be integrated and parallelized rather than continuing with the classic paradigm of ‘synthesize–generate a data point–synthesize again’ that is still in effect for drug discovery. The understanding of biological variables such as homo- and heterodimerization, alternative splicing, state-dependent conformations, posttranslational modifications, and disease-dependent gene and protein expression offer a myriad of exciting opportunities for the discovery and development of novel therapeutics.

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## DISCLOSURE/CONFLICT OF INTEREST

All authors declare that they are full time employees and as a result, shareholders of Neurocrine Biosciences Inc., a biopharmaceutical company focused on neurological and endocrine diseases and disorders. Further information is available through the Company’s website: [www.neurocrine.com](http://www.neurocrine.com).

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