Pharmacology and Therapeutics

GABAkines – Advances in the Discovery, Development, and Commercialization of Positive Allosteric Modulators of $GABA_A$ Receptors[1]

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Abstract (257 words)

Positive allosteric modulators of γ-aminobutyric acid-A ($GABA_A$) receptors or GABAkines have been important and highly used medicines for over 70 years for anxiety, epilepsy, sleep, and other disorders. Traditional GABAkines like diazepam have safety and tolerability concerns that include sedation, motor-impairment, respiratory depression, tolerance and dependence. Multiple GABAkines have entered clinical development but the issue of side-effects has not been fully solved. The present review focuses on the new GABAkines in development as well as several compounds in early preclinical development. The compounds that are presently being developed and commercialized include several neuroactive steroids (an allopregnanolone formulation (brexanolone), an allopregnanolone prodrug (LYT-300), Sage-324, zuranolone, and ganaxolone), the α2/3-preferring GABAkine, KRM-II-81, and the α2/3/5-preferring GABAkine PF-06372865 (darigabat). The neuroactive steroids are in clinical development for postpartum depression, intractable epilepsy, tremor, status epilepticus, and genetic epilepsy disorders. Darigabat is in development for epilepsy and anxiety. The imidazodiazepine, KRM-II-81 is in the late preclinical phase, is efficacious in animal models for the treatment of epilepsy and post-traumatic epilepsy, acute and chronic pain, as well as anxiety and depression. The efficacy of KRM-II-81 in models of pharmacoresistant epilepsy, preventing the development of seizure sensitization, and in brain tissue of intractable epileptic patients bodes well for improved therapeutics. Medicinal chemistry efforts are also ongoing to identify novel and improved GABAkines. The data document gaps in our understanding of the molecular pharmacology of GABAkines that drive differential pharmacological profiles, but emphasize advancements in the ability to successfully utilize $GABA_A$ receptor potentiation for therapeutic gain in neurology and psychiatry.

Key Words: GABAkines, neuroactive steroids, KRM-II-81, darigabat, epilepsy, anxiety, depression, pain

Running Title: New age GABAkines

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Abbreviations

CNS, central nervous system

β-CCT, beta-carboline-3-carboxylate-t-butyl ester

CGS 9896, 2-(4-Chlorophenyl)-1,2-dihydro-3h-pyrazolo[4,3-c]quinolin-3-one

DOV-51892, 7-(2-chloropyridin-4-yl)pyrazolo-[1,5-a]-pyrimidin-3-yl](pyridin-2-yl)methanone

GABA, γ-aminobutyric acid

$GABA_AR$, $GABA_A$ receptor

GABAkine, a positive allosteric modulator of GABAARs

iGlu, ionotropic glutamate

mGlu, metabotropic glutamate

NS11821 – structure not publicly disclosed

PAM, positive allosteric modulator

THIP, 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (gaboxadol)

QH-II-66, 7-Ethynyl-1-methyl-5-phenyl-1,3-dihydro-benzo[e][1,4]diazepin-2-one

XHe-II-053, ethyl 8-ethynyl-6-phenyl-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate

I. Introduction.

Physiology and behavior are modulated by the nervous system through a tightly regulated balance of excitatory and inhibitory processes. Multiple neurotransmitters participate in this intricate control network where the primary excitatory neurotransmitter in the mammalian nervous system, glutamate, is offset by the principal inhibitory neurotransmitter, γ-aminobutyric acid or GABA (**Fig. 1**). These balancing processes of excitation and inhibition work in concert to finely tune physiological and behavioral function.

The importance of GABA receptor modulation for medical practice is highlighted by the large number of GABA receptor modulators that have been and continue to be highly valued therapeutic agents. The present review focuses on the positive allosteric modulators of GABA (known as GABAkines) that have recently been commercialized or are in development for the treatment of neurological and psychiatric disorders. Some of the newer developments in this pharmacological space will also be touched upon. For some of the historical compounds that had been commercialized or were in development, the literature back to 2016 is reviewed to bring the reader up-to-date on newer work. Since diazepam and alprazolam are still highly used medicines, only the important new literature will be highlighted back to 2019.

Upon its release, GABA activates two classes of GABA receptors, $GABA_A$ and $GABA_B$ that differ in structure, biophysical properties, and pharmacology. $GABA_A$ (ionotropic) receptors ($GABA_ARs$) are rapidly responding ligand-gated ion channels belonging to the Cys-loop super-family (Hevers and Lüddens, 1998; Miller and Smart, 2010). This family, also includes nicotinic acetylcholine, glycine and serotonin subtype 3 receptors, is characterized by a loop formed by 13 highly conserved amino acids between two cysteine (Cys) residues (Miller and Smart, 2010; Thompson et al., 2010), and the formation of pentameric subunit complexes that form a central ion pore (Sine and Engel, 2006). The binding of GABA to the orthosteric binding site on the extracellular domain of $GABA_ARs$ receptors opens permeability to chloride ions with a high degree of selectivity against other anions (Kaila and Voipio, 1987; Sigel and Steinmann, 2012). The direction of flow is, in most cases, from outside to inside the neuron, resulting in hyperpolarization and reduced neuronal excitability. In addition, the increased conductance can result in shunting inhibition and modulation of neuronal gain (Chance et al., 2002; Mitchell and Silver, 2003; Prescott and Koninck, 2003; Mody and Pearce, 2004). However, depolarizing $GABA_AR$ currents have been reported in immature neurons, and under pathological conditions (Coull et al., 2005) with a proposed role in development and in neuroplasticity (Ben-Ari et al., 2007; Deidda et al., 2015; Ohtawa et al., 2017). In addition to its synaptic localization, where they mediate phasic transmission, $GABA_ARs$ are also present extrasynaptically where they are activated by low concentrations of ambient GABA and mediate tonic inhibition (Farrant and Nusser, 2005; Belelli et al., 2009).

Slow acting $GABA_B$ (metabotropic) receptors are similar to metabotropic glutamate receptors, members of the class C, G protein-coupled receptor family (Kaupmann et al.,

1997). The receptors are mandatory heterodimers of $GABA_B1$ and $GABA_B2$ subunits with each subunit composed of an extracellular Venus Flytrap domain and a transmembrane domain of seven α-helices (Mao et al., 2020; Papasergi-Scott et al., 2020; Park et al., 2020; Shaye et al., 2020). Recent cryogenic electron microscopy studies have helped resolve multiple conformations of the receptor, providing insight into receptor function and pharmacology (Mao et al., 2020; Park et al., 2020; Shaye et al., 2020). $GABA_B$ receptors act through activation of pertussis toxin-sensitive G proteins (Knight and Bowery, 1996) resulting in a prolonged decrease in neuronal excitability through the inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels, as well as the opening of G protein-coupled inward rectifying K^+ channels (Bettler et al., 2004; Gassmann and Bettler, 2012). $GABA_B$ receptors have been implicated in pathophysiological mechanisms of multiple neuronal processes and have been proposed as a drug target for a range of disorders including spasticity, pain, cough, bladder dysfunction, drug addiction, and epilepsy (Enna, 1997; Kumar et al., 2013). Substantial effort has been devoted to the discovery of compounds for $GABA_B$ receptors, but they have generally suffered from lack of efficacy, non-drug-like pharmacokinetic profiles, and side effects at sub-efficacious doses (Evenseth et al., 2020). Despite its poor brain penetration and rapid tolerance development, baclofen remains the only approved drug targeting $GABA_B$ receptors, with its clinical use as an antispasmodic agent and as a muscle relaxant (Ertzgaard et al., 2017).

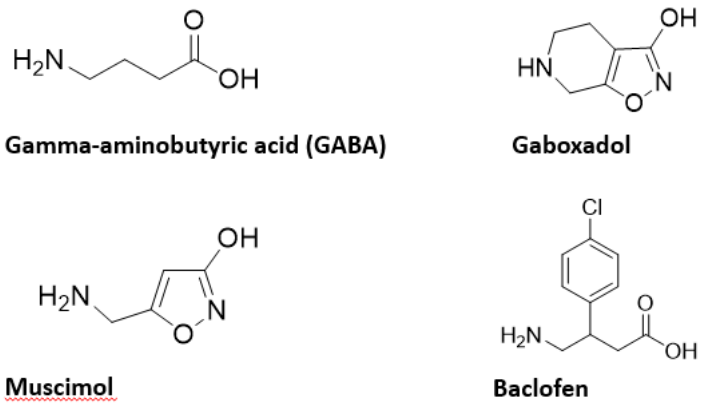

Figure 1. Structures of GABA and the direct-acting $GABA_A$ receptor agonists muscimol, gaboxadol (also known as THIP), and baclofen.

The primary excitatory neurotransmitter in the mammalian nervous system, glutamate, initiates excitatory changes in local neuronal circuits through binding to glutamate receptors. Glutamate receptors exist as both ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). IGluRs regulate synaptic neurotransmission through the gating of ions through an ion pore whereas mGluRs regulate slower transmission through coupling to G proteins (Schoepp, 1994; Nicoletti et al., 1996; Conn and Pin, 1997). Three types of ionotropic glutamate receptors have been identified that are

differentiated by structure and pharmacology: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors (Collingridge and Lester, 1989). mGluRs are likewise classified based upon their distinct family structures and the compounds which regulate their function. mGluRs are classified into three families: type I receptors include mGluR1 and mGluR5 that stimulate the phosphoinositide biochemical pathway; type II receptors include the mGluR2 and mGluR3 receptors; and type III receptors include GluR4, mGluR6, mGluR7, and mGluR8 receptors. Activation of the type II and type III mGluRs inhibits adenylyl cyclase (Schoepp, 1994; Nicoletti et al., 1996; Conn and Pin, 1997).

The interplay of glutamatergic excitatory and GABAergic inhibitory neuronal processes has dominant oversight of neurological and psychological function. For example, in 2019, (*S*)-ketamine (Spravato) was approved for use as an adjunct treatment for treatment-refractory depression. Ketamine is the first compound in the new class of drugs known as rapid-acting antidepressants (Witkin et al., 2019b). In contrast to the selective serotonin reuptake inhibitor antidepressants that generally take weeks of daily dosing to produce a response (Katz et al., 1996, 2004), ketamine can transduce immediate relief from symptoms of major depressive disorder and can do so in patients that have been otherwise refractory to standard of care antidepressants (Zarate et al., 2006). In regions such as the hippocampus, glutamate release from pyramidal cells is kept in check through the activity of fast-spiking GABA inhibitory neurons. Ketamine, like its congener, phencyclidine (Homayoun and Moghaddam, 2007), acts by blocking the NMDA ion-channel and reducing the GABA-driven inhibitory tone on pyramidal cells, thus enabling the efflux of glutamate (Thelen et al., 2019). Free glutamate is then available to further the cascade of rapid-acting antidepressant response by binding to AMPA receptors (Alt et al., 2006) (**Fig. 2**). However, blockade of NMDA receptors by ketamine (Anis et al., 1983) is also an initiator of the characteristic dissociative and psychomimetic actions of ketamine (Nicholson and Balster, 2003). Understanding the inhibitory and excitatory balances involved in therapeutic actions and side-effects of compounds enables clues to creating rapid-acting antidepressants devoid of ketamine-like side effects (Witkin, 2020). In a similar fashion, such an understanding provides new directions for other therapeutic areas as well.

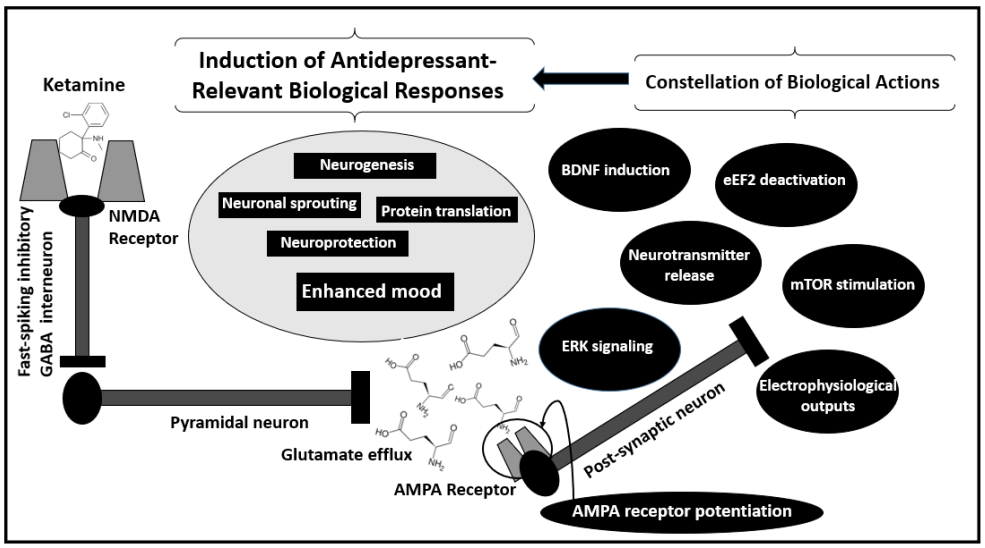

Figure 2. An example of GABA – glutamate dynamics. A simplified postulated mechanism of action of the rapid-acting antidepressant ketamine. Ketamine binding to the NMDA receptor ion channel activates fast-spiking inhibitory GABA neurons whose activity serves to disinhibit pyramidal neurons, resulting in glutamate release. Glutamate binds to AMPA receptors on postsynaptic neurons results in AMPA receptor amplification, initiating a biological cascade in a host of intermediary biology pathways that are associated with the antidepressant responses to ketamine. eEF2: eukaryotic elongation factor 2; ERK: extracellular signal–regulated kinase; mTOR: mammalian target of rapamycin. From Witkin et al. (2019b) with permission of the publisher.

II. $GABA_A$ Receptors

As with the $GABA_B$ receptors, $GABA_A$ receptors ($GABA_ARs$) have been mechanistically linked to a host of pathophysiological processes including sleep, pain, epilepsy, depression, schizophrenia, etc. Discussion of these areas will be provided within the present review.

$GABA_ARs$ are a complex comprised of five subunits, which are expressed in humans as the following types: α1-6, β1-3, γ1-3, ρ1-3, δ, ε, π, and θ (Hevers and Lüddens, 1998; Olsen and Sieghart, 2009). Each functional $GABA_AR$ typically includes α-, β-, and γ or δ -subunits in a 2:2:1 ratio for functional activity. The pentameric chloride ion channel is composed of a large extracellular domain containing an orthosteric binding pocket, a transmembrane domain composed of the pore-lining M2 helices and a small intracellular domain which is a site for modulation by associated proteins (Chen and Olsen, 2007; Han et al., 2020). The orthosteric binding pockets occupy the interfaces of the α and β subunits which are typically two per pentamer (Phulera et al., 2018; Zhu et al., 2018; Laverty et al., 2019). The two binding pockets differ structurally (Masiulis et al., 2019), have different affinities for ligands, and act synergistically (Baumann et al., 2003). Binding of GABA, the endogenous agonist, induces local structural changes in the

binding pocket, which result in a concerted rotation of the five extracellular domains (Masiulis et al., 2019). Rotation of the extracellular domains is then translated into motion of the transmembrane domain resulting in a conformational change of channel activation and inactivation gates (Hevers and Lüddens, 1998; Masiulis et al., 2019).

Other agonists and antagonists can bind to the same orthosteric site but can produce different changes in both the binding pocket and in channel conformation (Masiulis et al., 2019). $GABA_A$Rs also contain numerous binding sites for modulators including anesthetics, barbiturates, neurosteroids (Olsen, 2018; Solomon et al., 2019; Vega Alanis et al., 2020), and benzodiazepines. Like the orthosteric site, the benzodiazepine receptor is located in the extracellular domain, but on the interface of α and γ subunits (Laverty et al., 2019; Masiulis et al., 2019). Binding of benzodiazepine ligands does not produce marked changes in the conformation of the extracellular domain but rather facilitates the actions of orthosteric ligands. It is noteworthy that different benzodiazepine receptor ligands have substantially different binding modes which may have an impact on the efficacy of compounds (Scott and Aricescu, 2019). For example, while full agonists of benzodiazepine receptors (diazepam, alprazolam) (**Fig. 3**) bind deep in the binding pocket (Masiulis et al., 2019) bretazenil, with partial agonism, and flumazenil with no activity, bind higher in the pocket (Miller et al., 2018; Scott and Aricescu, 2019).

The complexity of the subunit composition of $GABA_A$Rs in the CNS, together with distinct regional, cellular, and subcellular distributions, and diverse pharmacologic properties carries functional significance (Engin et al., 2018; Olsen and Sieghart, 2009; Siebert et al., 2018).

III. GABA and Therapeutics

Both positive and negative allosteric modulators of $GABA_A$Rs exist (Olsen, 2018). The current review focuses on positive allosteric modulators (PAMs), or GABAkines. Consistent with the naming of AMPA receptor potentiators as AMPAkines, we are using the term GABAkines to denote positive allosteric modulators of $GABA_A$Rs. The dominant role of GABA as an inhibitory neurotransmitter in the mammalian nervous system provides two *a priori* predictions regarding a role for GABA in therapeutics: 1) altering GABA neurotransmission should have pervasive actions on a host of neurological and psychiatric functions; and 2) alterations in GABA neurotransmission could be accompanied by GABA-related side effects. The data summarized below will illustrate the reality of both of these predictions. The discussion in the present review focuses on the enhancement of GABA neurotransmission by GABAkines. Since inhibitory neurotransmission through $GABA_A$Rs can also be augmented through the orthosteric binding domain, a brief discussion will also be given to these direct-acting $GABA_A$R agonists (**Section IV**).

The discovery, development, and clinical history of drugs that augment GABAergic neurotransmission is best illustrated by the work that was directed toward identifying non-sedating drugs to treat anxiety. This empirical adventure began long before a linkage of these drugs to GABA was uncovered. The first rationally-designed anxiolytic drug,

meprobamate (Miltown) (**Fig. 3**) was discovered and championed by Frank Berger who, with Bernard Ludwig, modified the muscle relaxant mephenesin with the goal of reducing muscle-relaxing and sedative properties while augmenting anti-anxiety effects. Berger argued that an antianxiety drug was needed in a cold-war environment. Miltown was the first blockbuster drug and was, in the late 1950s, being used by so many people in the United States that its name was used in the popular language of the times (Tone, 2009). However, the popularity of Miltown led to its demise with people reporting dependence, a fate to later impact the medical prescriptions for diazepam (Valium) as well (Tone, 2009). A similar drug from Berger's lab, carisoprodol, is a centrally-acting muscle relaxant used for the treatment of back pain. Carisoprodol (~25%) is metabolized by CYP2C19 to meprobamate (Dean, 2017).
The carbamate, meprobamate, led to the next generation of anxiolytic drugs - the 1,4-substituted benzodiazepines. In search of a drug to compete with meprobamate, Hoffmann La-Roche synthesized many compounds without finding improvement over meprobamate and the project was terminated by management. Months later, these compounds were slated for destruction when a lab technician noted that Ro 5-0690 had not been tested (Winters, 2016). The head of medicinal chemistry, Leo Sternbach, directed animal testing (Randall et al., 1960) and took the compound himself as well, providing the first clinical data on chlordiazepoxide (Winters, 2016). With the FDA approval of chlordiazepoxide (Librium) (**Fig. 3**) in 1959 and its introduction into clinical practice, another generation of anxiolytic agents was born and, as with meprobamate, found widespread use for the treatment of anxiety.

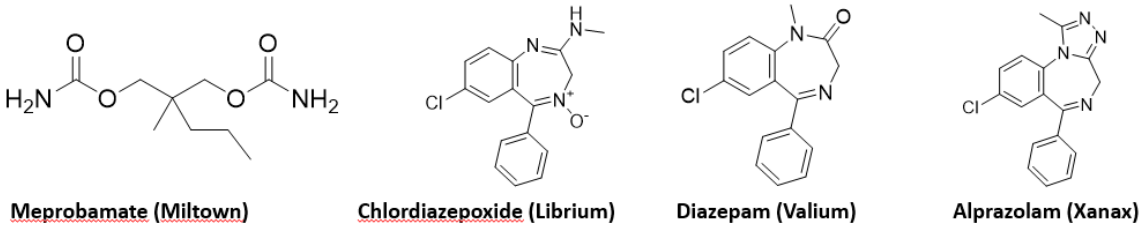

Fig. 3. Structures of first- and second-generation GABAkines used in the treatment of anxiety along with the currently widely prescribed drug alprazolam. Not shown here are barbiturates more prominently used as anxiolytics prior to the commercialization of the structures shown.

Diazepam (**Fig. 3**) arose from the 1,4-substituted benzodiazepine chemical series and was approved for clinical use in 1965. By 1970, antianxiety drugs, mostly benzodiazepines, were used by 1 in 5 woman and 1 in 13 men in the United States (Parry et al., 1973). Diazepam was and still is a highly valuable drug used for the treatment of anxiety and other disorders (Woods et al., 1992; Szarmach et al., 2017; Weintraub, 2017; McTague et al., 2018; Jafarpour et al., 2019). Despite its bad press for being addictive (Hollister et al., 1963), and the reluctance of the medical community to prescribe it wholesale, it is still widely used. Diazepam is on the List of Essential Medicines of the World Health Organization. In the United States, diazepam as an anxiolytic has been largely supplanted by another benzodiazepine, alprazolam (Xanax) (**Fig. 3**) (Aden and Thein, 1980;

Maletzky, 1980) likely for reasons of the highlighting of diazepam's addiction and dependence potential.

Benzodiazepines such as diazepam have some potential drawbacks, depending on their intended use. For example, when used as an anxiolytic, undesirable sedative effects at therapeutic doses can cause motor impairment. For sleep induction, however, sedation is desired. Memory impairment is another side-effect that is often unwanted. Like many CNS-acting drugs, benzodiazepines can produce tolerance to certain of its therapeutic effects, and induce physical dependence, and abuse (Woods et al., 1992). It should be noted that tolerance to the anxiolytic effects of these drugs has been difficult to demonstrate (Margules and Stein, 1968; Laughren et al., 1982).

Several additional studies exploring novel pharmacology and potential novel therapeutic applications have been published on these key compounds since 2016 and a summary is provided below.

A. Meprobamate

An overview of the pharmacology of meprobamate in comparison with the newer carbamates is available (Löscher et al., 2021). New findings using machine-learning technologies,) analyzed drug properties to deduce that meprobamate would have antifungal properties colleagues (Udrescu et al., 2020. These findings were subsequently analyzed by molecular docking. Kumar and Dillon (2016) provided an intriguing molecular pharmacology assessment of the muscle relaxant carisoprodol and its major metabolite, meprobamate. The over-lapping and unique actions of meprobamate suggested to these authors that additional structure activity relationship be conducted to more fully exploit the therapeutic potential of these carbamates.

B. Chlordiazepoxide

A relatively recent overview of this compound is available (Ahwazi and Abdijadid, 2021). Recently, combinations of clidinium with chlordiazepoxide were shown to be effective in patients with functional dyspepsia that were refractory to treatment by proton pump inhibitors (Puasripun et al., 2020). Librax® is a marketed formulation of this drug combination. The comparative clinical pharmacology of chlordiazepoxide and lorazepam in the treatment of alcohol withdrawal demonstrated that both were able to control delirium tremens, but, it was suggested that chlordiazepoxide might be somewhat less efficacious in overall symptom control. Szarmach et al. (2017) provided an analysis of best practices for use of adjunctive benzodiazepine anxiolytics in combination with antipsychotic agents in the control of positive and negative symptoms in schizophrenic patients.

C. Diazepam

An overview of the pharmacology of diazepam has been recently published (Dhaliwal et al., 2021In preclinical studies using an inventive methodology, Leonard and Kangus

(2020) evaluated the possible opioid-sparing (ability to reduce the amount of opioid needed to produce an antinociceptive effect) effects of diazepam. Diazepam had no anti-nociceptive actions of its own and had very limited ability to augment the effects of oxycodone. In addition, Pilipenko et al. (2019) reported on the potential favorable use of diazepam in a rat model of pre-dementia.

New delivery systems for diazepam have been studied including a nasal spray (Valtoco®,) rectal and intravenous forms (Cornett et al., 2021). Rogawski and Heller (2019) compared the activity of a buccal film with the rectal gel (Diastat®) and concluded that the buccal form is a valuable consideration for use in out patient settings.

Arelatively recent report of a meta-analysis of randomized controlled trials indicated that i.v. lorazepam was better than i.v. diazepam for the cessation of status epilepticus in adults (Kobata et al., 2020). This group also reported results of a study comparing low dose diazepam to low dose sodium valproate in the treatment of febrile convulsions, and documented a potentially improved side-effect profile of sodium valproate. Afzalimoghaddam et al (2021) compared diazepam with midazolam (both i.v. in conjunction with fentanyl) in patients undergoing reduction of shoulder dislocation,and diazepam was deemed superior to midazolam (time to muscle relaxation, time to reduction, and satisfaction of both patient and physician). The misuse of benzodiazepine anxiolytics in patients with alcohol use disorder along with psychiatric comorbidities were studied in adult hospitalized patients (Lopez et al., 2021). Diazepam was more misused in this patient population than other benzodiazepines and anxiety/depression were the most common comorbidities among patients that were misusing benzodiazepines.

D. Alprazolam

A recent review of this drug was published (George and Tripp, 2021). Updatesto the underlying mechanisms of action of alprazolam have reported involvement of CB_1 receptors in its anxiolytic-like effects in rodent models Batista and Moreira (2019).

Prescribing practices of dentists in the U.S. (2013-2018) showed that diazepam was the most frequently prescribed with alprazolam and others second (Teoh et al., 2021).

IV. Direct-Acting Agonists

Inhibitory neurotransmission can be augmented through $GABA_AR$ ligands interacting with either orthosteric or allosteric domains on the $GABA_AR$ complex. Orthosteric agonists, like GABA itself, are also called direct-acting agonists. Muscimol and a structural analog, gaboxadol or THIP, are two common examples (**Fig. 1)**. Gaboxadol is a potent agonist of GABA receptors that contain α_4, α_6, and δ, subunits, which have more restricted anatomic distribution in the thalamus, hippocampus, and cerebellum and are mainly extrasynaptic in location.

Attempts to capitalize on direct-acting $GABA_AR$ agonists for therapeutics in multiple areas of GABA insufficiency have been made, but due to a general lack of efficacy and GABA-associated side effects, these agents are not in general clinical use. A small trial with gaboxadol in patients with Huntington's disease was conducted over a period of two weeks. Like muscimol, maximal doses of gaboxadol produced somnolence, unsteadiness of gait, and reduced attention to sensory stimulation. Despite these indicators of enhanced GABAergic impact, gaboxadol did not improve motor function or cognitive performances (Foster et al., 1983). Gaboxadol has also been considered as a medication to promote sleep (Wafford and Ebert, 2006). Ovid Therapeutics recently conducted a Phase-3 clinical trial of gaboxadol (OV-101) for treatment of Angelman Syndrome (Rakhit, 2020) and anticipated development for fragile X chromosome. Gaboxadol, however, failed to meet the primary endpoint for treatment of Angelman Syndrome and further development was suspended (Bryson, 2020). Heiss and colleagues (Heiss et al., 2019) have recently explored the potential of muscimol as a potential treatment for pharmacoresistant epilepsy. Although there were some effects of treatment, the data taken as a whole did not substantiate the efficacy of muscimol when administered intracerebrally.

V. GABAkines

A. History. Much as the term AMPAkine has been coined to refer to drugs that act as positive allosteric modulators at the AMPA glutamate receptor, we have chosen to use the term GABAkine to refer to drugs that act as positive allosteric modulators at $GABA_ARs$. Although GABAkines have existed for years (see historical discussion below), it was not until the 1970s that GABA was implicated in their pharmacological actions. Traditional medicine utilized a host of hypnotic and sedative agents (e.g., ethanol, opioids, cannabinoids) with a transition to the use of paraldehyde, chloral hydrate, and bromides at the end of the 19th century. Barbiturates were initially synthesized by Adolf von Baeyer in 1864 and were introduced to the market as sedatives, hypnotics and antiepileptics in the early 20th century (López-Muñoz et al., 2005). Their use was rapidly expanded by the introduction of approximately 50 barbiturates, producing diverse clinical profiles but narrow therapeutic windows which created the potential for overdose, abuse, dependence and for exacerbation of seizures upon withdrawal (Isbell, 1950). The mechanism of action of barbiturates is not completely understood and modulation of multiple voltage-gated and ligand-gated ion channels has been proposed (Löscher and Rogawski, 2012). The main modulatory effect is, however, through modulation of $GABA_ARs$ with potentiation at low concentrations and direct activation at higher concentrations, pharmacological properties that decrease the safety of barbiturate GABAkines relative to the benzodiazepine GABAkines. Inter-subunit binding sites in the transmembrane domain of the human $GABA_AR$ were identified (Chiara et al., 2013) but precise binding domains and their structural impact upon binding remain to be fully characterized (Scott and Aricescu, 2019). The medical use of barbiturates declined in the 1950s after the introduction of meprobamate and the 1,4-benzodiazepines.

From the era of meprobamate and the benzodiazepines, there have been many new GABAkines discovered and some of these advanced into clinical development. Although the present review focuses on the newest GABAkines in development, some historical preface will be provided. The primary therapeutic areas of interest for these compounds were anxiety and epilepsy. However, other areas of interest were explored as well.

B. Anxiolytic Activity and Sedation. One issue with the benzodiazepine anxiolytics that is key to understanding their therapeutic value as well as an aspect of their pharmacology that impedes therapeutic utility is dose-dependent sedation. While sedation is sometimes desired, as in sleep induction, it is a dose-limiting side-effect for the majority of therapeutic applications. For example, although it is well-known that increasing inhibitory tone in the nervous system by amplifying GABA signaling is a critical mechanism for many neurological and psychiatric disorders, the 1,4-benzodiazepines are often not used because efficacious plasma levels cannot be achieved without undesirable sedative and motor-impairing effects.

From the standpoint of both efficacy and side-effect liabilities, improvements in standard-of-care medicines for the symptomatic relief of anxiety are needed. Some antidepressant drugs, in particular, the selective serotonin reuptake inhibitors, are used for the treatment of anxiety (Bandelow, 2020). Although these compounds are generally not sedating, they bear other side effects such as treatment-emergent weight gain and sexual dysfunction (Atmaca, 2020; Gill et al., 2020). Moreover, their anxiolytic actions, like their effects on depression (Katz et al., 2004), require weeks of daily dosing (Cvjetkovic-Bosnjak et al., 2015). Due to such limitations, benzodiazepine anxiolytics continue to be used for the acute treatment of anxiety. Drugs like alprazolam are widely prescribed but come with the burden of sedation and motor-impairment, as well as abuse and dependence potential (Străulea and Chiriţă, 2009; Reissig et al., 2015; Duke et al., 2020). Moreover, drugs like alprazolam can facilitate the suppression of respiration that has led to emergency room visits and deaths (Jann et al., 2014; Hedegaard et al., 2018; Witkin et al., 2019a).

Rational drug discovery efforts directed at creating improved GABAkines came from basic pharmacological data along with the discovery of the benzodiazepine receptor (Möhler and Okada, 1977; Squires and Brastrup, 1977) and its role in potentiation of GABA currents by GABAkines (Choi et al., 1977))(see historical overviews by (Schallek et al., 1979; Tallman et al., 1980; Haefely, 1989)). This discovery enabled establishment of binding assays (Williamson et al., 1978), with promising ligands evaluated in animal models for efficacy and reduced unwanted side effects (reviewed in Skolnick, 2012).

Multiple compounds progressed into clinical trials due to their favorable preclinical profile including bretazenil, abecarnil, alpidem, and ocinaplon (**Fig. 4**). Alpidem was approved as an anxiolytic with relatively little sedation (Musch et al., 1988) but was later withdrawn due to high occurrence of hepatitis (Baty et al., 1994). Ocinaplon demonstrated anxiolytic effects without sedation (Lippa et al., 2005; Czobor et al, 2010), but was later withdrawn from further development due to concern over incidents of liver enzyme elevation.

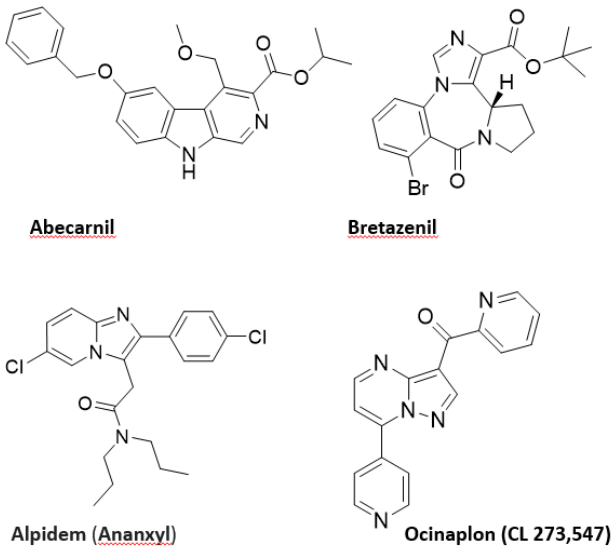

Fig. 4. Structures of some compounds with anxio-selective profiles that entered into clinical investigation.

Only a limited number of more recent studies have been published on these key compounds back to 2016. A study with abecarnil in photosensitive epileptic patients showed efficacy with some sedation (Kasteleijn-Nolst Trenité et al., 2016). Bretazenil was investigated by Neugebauer et al. (2018) showing brain region specific areas where GABAkines are likely to produce memory impairments; these data are a caution to the idea of rescuing deficits in schizophrenia with GABAkines. An evaluation of the pharmacological therapies that have been used for the management of benzodiazepine withdrawal was published without solid conclusions due to a host of limitations of the data base (Baandrup et al., 2018).

There have been no new published reports on ocinaplon since the data on the discriminative stimulus effects in rats in 2011 (Vinkers et al., 2011).

Initial pursuit of the ideal GABAkine had been directed toward the creation of a non-sedating anxiolytic (see Skolnick, 2012 for reviewThe potential for the discovery process to proceed on rational grounds was first given hope by Lippa and colleagues who postulated that there were $GABA_A$Rs that drive sedation more than other $GABA_A$R; furthermore, that there existed $GABA_A$Rs that were more specifically utilized to generate anxiolytic effects than others (Lippa et al., 1978; Klepner et al., 1979). Based upon the ability of some compounds to produce anxiolytic-like effects without sedation in animal models, it was hypothesized that multiple benzodiazepine receptors exist and that some mediate anxiolytic effects and others sedation (Klepner et al., 1979; Lippa et al., 1981;

Lippa et al., 1982). The non-sedating anxiolytic-like compound, CL218,872 (**Fig. 5**) was the first to jump start this idea.

The advent of molecular biology enabled further refinement to the search for anxio-selective drugs. The concept of benzodiazepine type 1 and type 2 receptors (Lippa et al., 1978, 1981, 1982; Klepner et al., 1979) was integrated into the current understanding of the structure and function of the $GABA_AR$ (**Section II**). Basic and applied research in this area focused on the specific α-subunit comprising the $GABA_AR$ assembly since modifications of this subunit produced major changes in pharmacological activity. Genetic, pharmacological, and behavioral evidence was used to suggest that α1-subtype-containing $GABA_ARs$ preferentially mediate the sedative, amnestic, and ataxic effects of ligands, as well as dependence (Rudolph et al., 1999; McKernan et al., 2000; Wafford, 2005; Licata et al., 2009; Ator et al., 2010; Tan et al., 2010), whereas α2- and α3-subtypes mediate anxiolytic effects (Löw et al., 2000; Rudolph and Möhler, 2014) and pain therapeutics (Dias et al., 2005; Lewter, 2019); the α5-subtype has been implicated in memory function (Collinson et al., 2002; Dawson et al., 2006). Based primarily on the data associating α1-containing $GABA_ARs$ receptors with sedation, discovery efforts over the last 15 years have been directed at the identification and development of GABAkines as anxiolytics, antiepileptics and analgesics with preference for α2- and α3- relative to α1-containing $GABA_ARs$ (Rudolph and Knoflach, 2011) (**Fig. 5**).

It is important to note that the α1 hypothesis has been challenged by both animal and human pharmacological data. For example, ocinaplon (**Fig. 4**), produced no sedation at the dose that produced significant anxiolytic effects, despite having higher efficacy at potentiating currents evoked in α1 $GABA_ARs$ than α-2, -3 and -5-containing $GABA_ARs$ (Lippa et al., 2005). And, as reviewed later, the α1-sparing, subtype-selective GABAkines showed more sedation than predicted from their pharmacological profiles in the preclinical studies. As discussed, (Skolnick, 2012), there continues to be a great deal unknown about the structural determinants of specific $GABA_ARs$ that correspond to specific efficacy and side-effects. This uncertainty is exacerbated by the complexity of behavioral endpoints, specific $GABA_AR$ localization, and pharmacology.

One of the first such molecules investigated was L–838,417 (**Fig. 5**), a partial agonist at α2,3- and α5-containing $GABA_ARs$ and a negative allosteric modulator at α1-containing receptors. L-838,417, produced anxiolytic-like effects in the elevated plus maze but did not impair motor activity (McKernan et al., 2000; Carling et al., 2005). Further drug discovery efforts resulted in three compounds which progressed into clinical studies; two analogs of L–838,417 (TPA-023 and MRK-409) and a structurally unrelated compound, TPA-023B (**Fig. 5**) (Atack, 2011). All three compounds were partial agonists at α2/3 subtypes with no substantial efficacy at α1-containing $GABA_ARs$ in vitro (Atack et al., 2006); they were all efficacious in animal models of anxiety without observed sedation (Atack, 2009). Two more recent α1-sparing, subtype-selective GABAkines have also been brought forward: NS11821from Neurosearch (structure not disclosed) and AZD7325 from Astra Zeneca.

Despite preclinical hopes , clinical data on these compounds presented a more complex picture. All of these compounds produced more sedation, dizziness, drowsiness, and motor incoordination than was hoped (see (de Haas et al., 2007, 2008, 2012; Atack, 2009; Atack et al., 2010; (Fujita et al., 1999)). NS11821and AZD7325 (**Fig. 5** also exhibited more dizziness, somnolence, and sedation in humans than hoped for but were otherwise well-tolerated (Zuiker et al., 2016) (Chen et al., 2014; Jucaite et al., 2017). For example, in the study by (Zuiker et al., 2016), NS11821 is a partial $GABA_A$ agonist with relatively dominant α2,3 and α5 subtype efficacy but negligible α1 agonism. This first-in-human study was performed in healthy male subjects using a single-dose, parallel, double blind, placebo-controlled, randomized, dose-escalation study design. In total six cohorts (N=48) were enrolled. The eight subjects of each cohort received NS11821 (10 mg, 30 mg, 75 mg, 150 mg, 300 mg or 600 mg) or placebo in a 6:2 ratio. At low dose levels, NS11821 had a relatively low exposure and a more-than-proportional increase of the area under the curve and maximum plasma concentrations, probably due to poor solubility. Saccadic peak velocity decreased in a dose-related manner while limited impairments were seen on body sway and the visual analogue scale for alertness. The most common adverse events were somnolence and dizziness, which were more prominent with the higher doses. Although no positive control was used in this study, the results were compared post hoc with a Centre for Human Drug Research dataset for lorazepam 2 mg. The maximum saccadic peak velocity effects seemed comparable to the typical effects of lorazepam, whereas the other central nervous system effects were smaller. These results support the pharmacological selectivity of NS11821 and show that pharmacodynamic effective doses of NS11821 were safe and well tolerated in healthy subjects.

None of these compounds has yet progressed to become a medicine. There are many reasons for a drug not achieving the difficult milestone of becoming an approved therapeutic. The present review will not address this complex topic. For subtype selectivity discussions, the commentary by Skolnick (2012) is recommended. It is also well known that translation from the discovery lab to patient therapeutics has a host of obstacles including seemingly small ones. For example, Yamamura's lab showed as early as 1979 that the binding of compounds to benzodiazepine receptors is temperature sensitive (Speth et al., 1979). Along a chain of predictive events, the smallest error can lead to a disconnect in biological integrity.

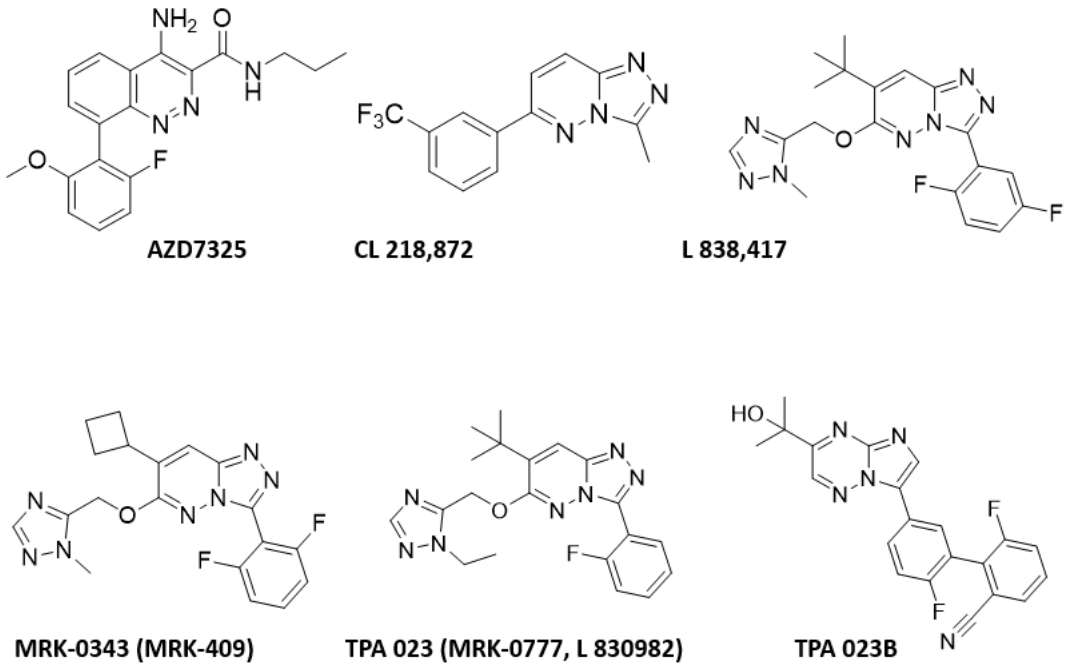

Fig. 5. Structures of some early compounds developed for their reduced potentiation of $GABA_A$ receptors containing α1 protein subunits. The structure of NS11821 has not been publicly disclosed.

Within last four years L834,417 has been the subject of several studies. Wang et al. (2018) used this compound to demonstrate full substitution for the discriminative stimulus effects of propofol and concluded that α 5-containing $GABA_ARs$ are a prominent mechanism of action. L838,417 was also utilized in modeling the pharmacophore for driving potency and efficacy of $\alpha_{1,2,3,5}\beta\gamma_2$ $GABA_A$ receptor interactions where the α_1-Gly^{201}/α_3-Glu^{225} appears to be pivotal (Söderhielm et al., 2018).

Another study utilized L834,417 along with other GABAkines to restore inhibitory tone (and therapeutic relief) in a mouse model mouse model of Alzheimer's disease that demonstrates age-dependent accumulation of Aβ, neuroinflammation, and neurodegeneration (Petrache et al., 2019). A role for α2/3-containing $GABA_ARs$ in the development of gastric function of rats made use of L834,417 as a selective research tool (Clyburn et al., 2019). Third ventricle doses of L838,417 stimulated enhanced sucrose consumption; these data helped to establish that α2/3-containing receptors but not α1- are the principal drivers of benzodiazepine-induced augmentation of taste palatability (Nelson et al., 2019). L838,417 was utilized as a partial agonist targeting α5-containing $GABA_ARs$ to help interrogate a role for these receptors in fibromyalgia-modeled pain in rats (De la Luz-Cuellar et al., 2019). In rats, L838-417 did not affect social behavior or attention (Paine et al., 2020). In an elegant series of experiments, Lorenzo et al. (Lorenzo et al., 2020) used L838,417 as a tool to help dissect the α2-$GABA_AR$ basis for regulation of neuropathic pain. The low abuse potential of this compound was shown by its lack of

self-administration in Rhesus monkeys (Huskinson et al., 2019; Berro et al., 2021); triazolam and lorazepam were self-administered (Berro et al., 2021).

The potential value of augmenting α2/3-containing $GABA_A$Rs in schizophrenia was suggested by a preclinical study with rats in which TPA 023 was found to moderate some deficits in a rat model (Rajagopal et al., 2018). TPA 023B was utilized as one of the tools to help define α2- and α3-containing $GABA_A$Rs in the regulation of itch (Ralvenius et al., 2018). Zeilhofer and colleagues also reported that TPA-023B suppresses the affective component (tonic) of pain (Neumann et al., 2021).

Even though benzodiazepines, as a class, act at all γ subunit containing $GABA_A$Rs (α1, 2, 3, and 5), some compounds produce less sedation than others. One such compound is the 1,5 benzodiazepine clobazam (Wildin et al., 1990; Sankar, 2012), whose milder sedative liability could have contributed to its approval as an add-on therapy for Lennox-Gastaut syndrome (Ng et al., 2011). A small proof of concept clinical trial also reported reduction of capsaicin-induced hyperalgesia with clobazam (Besson et al., 2015). The activity of clobazam might be due, at least in part, to its active metabolite, N-desmethyl-clobazam. N-desmethyl-clobazam exhibits functional selectivity for α2/3/5-containing GABAARs where it is less efficacious at α1-containing receptors (Ralvenius et al., 2016). N-desmethyl-clobazam produced significant analgesia in rodent models without sedation (Ralvenius et al., 2016). The authors of the study filed a patent for the clinical use of N-desmethyl-clobazam for chronic pain (Ralvenius et al., 2016). A Phase-1 clinical trial with N-desmethyl-clobazam has been completed and the data published (Matthey et al., 2020); in this study, N-desmethyl-clobazamproduced no sedation, but there was also no significant efficacy in reversing hyperalgesia. N-desmethyl-clobazam has been registered for a Phase-2 clinical trial for treatment of peripheral neuropathic pain (Besson, 2020) with expected completion mid-2021.

C. Epilepsy.

Many first-generation antiepileptic drugs have primary actions as GABAkines. These include valproic acid (Depakene), clorazepate dipotassium (Tranxene), clonazepam (Klonopin), diazepam (Valium), phenobarbital (Luminal), and primidone (Mysoline) (Gitto et al., 2010), some of which are still in use for the treatment of epilepsy (Gitto et al., 2010). For example, valproate is a GABAkine with use in generalized seizures and status-epilepticus (Rahman and Nguyen, 2020; Liampas et al., 2021). GABA-related side effects of dizziness and somnolence are induced and for which discontinuation of medication can occur (Rahman and Nguyen, 2020). Valproate can be contraindicated in the elderly epileptic patient despite the fact that it is sometimes used in geriatric dementia (Liu and Wang, 2020). Valproic acid is also used in bipolar disorder but its utility is limited due to deficits in affective processing and sustained attention (Holmes et al., 2008) and to the more severe negative impact on cognitive function than lithium (Xu et al., 2020). Other non-GABAkine antiepileptic drugs also suffer from these and other side-effects (Kowski et al., 2016).

Despite the continued use of some older generation GABAkines, improvements are needed in both efficacy and safety (Witkin et al., 2021). It is estimated that in about 70% of patients, existing drugs are not fully efficacious in controlling seizures (Marson et al., 2007; Sinha and Siddiqui, 2011; Banerjee et al., 2014). Despite being maintained on multiple antiepileptic medicines, many patients continue to have seizures (Błaszczyk et al., 2018). For these reasons, some patients elect to have invasive therapeutic procedures such as surgical resection or disconnection (Adelson, 2001; Hwang and Kim, 2019). Pharmacoresistance to anticonvulsant therapy continues to be one of the key obstacles to the treatment of epilepsy (Franco et al., 2014). A very recent review highlights the GABAkines that are being considered for focal epilepsy (Janković et al., 2021).

D. Other Therapeutic Indications.

Since GABA is a pervasive and primary neuroinhibitor, GABA influences other biological functions in addition to anxiety and seizure activity. As such, GABAkines have been considered as potential therapeutic modulators for diverse disorders including schizophrenia, pain, depression, anxiety, cognition, and disorders of cardiovascular function. A recent review is highly informative (Maramai et al., 2020). Since other compounds and indications are reviewed elsewhere in the current manuscript, this section will provide a very brief summary of the current focus of agents acting at $GABA_A$Rs that have α4, α5, or α6 subunit composition because: 1) the therapeutic potential of agents acting at these receptors is less defined than those acting a α1-, α2, and α3-containining $GABA_A$Rs discussed more thoroughly across this review; and 2) because ther therapeutic potential of such compounds as defined to date has generally been distinct from that of the other GABAkines.

Early work on GABAkines that specifically amplified GABA signaling through α5-containing $GABA_A$Rs identified potential therapeutic usefulness in the areas of schizophrenia and airway muscle relaxation (c.f., (Clayton et al., 2015)) (see reviews by Jacob, 2019; Mohamad and Has, 2019). Current work outlining a rationale for the potential therapeutic value of these selective GABAkines has recently been published in the diverse areas of schizophrenia, pain, depression, anxiety, cognition, and cardiovascular function (c.f., (Batinić et al., 2017; Donegan et al., 2019; Hernández-Reyes et al., 2019; Prevot et al., 2019; Bojić et al., 2021; Davenport et al., 2021; Fee et al., 2021; Franco-Enzástiga et al., 2021); however, see (Xue et al., 2017)).α4-and α6-containing $GABA_A$Rs are not sensitive to typical GABA modulators (diazepam-insensitive) and their focus is beyond the scope of this review. It is important however to emphasize here, that α4β3δ assemblies are considered extrasynaptic and as such have key relevance to some of the newer advancing GABAkines (see **Section VIA**). α4-containing $GABA_A$Rs also might be relevant for control of breathing (Yocum et al., 2016).The prospect of a therapeutic impact of α6-containing $GABA_A$Rs has also not been neglected for multiple areas including pain and Tourette syndrome (c.f., (Huang et al., 1999; Chiou et al., 2018; Vasović et al., 2019; Tzeng et al., 2020; Cadeddu et al., 2021)).

VI. GABAkines in clinical development

The search for improved GABAkines has been directed toward achievement of efficacy at therapeutic levels that do not induce (unless specifically desired) sedation or sleep, but also that do not impair motor function or memory, and do not induce tolerance, dependence, or abuse. Another motive for the development of new GABAkines is the improvement of efficacy.

There are currently two classes of GABAkines in clinical development: several neuroactive steroids (**Fig. 6**) and two small molecules that have different degrees of selectivity for α2/3-containing $GABA_A$Rs (**Table 1**). In addition to compounds in development, this review will also briefly address ongoing efforts to identify new chemical scaffolds and novel GABAkines.

Table 1. GABAkines in clinical development.

Compound	Other Names	Pharmacological Class	Development Phase	Current Clinical Use or Investigation	Other Indications Investigated or Planned	Company
Allopregnanolone	Sage-547 Brexanolone Zulresso®	Neuroactive steroid	Marketed Ph2 Ph1a/2b	Post-partem depression FXTAS Alzheimer's disease	Refractory status epilepticus Essential tremor PTSD	Sage Therapeutics Inc
Sage-217	Zuranolone®	Neuroactive steroid	Ph2 Ph2	MDD Parkinson's disease	Bipolar disorder Essential tremor Insomnia model	Sage Therapeutics Inc
Sage-324		Neuroactive steroid	Ph2	Essential tremor		Sage Therapeutics Inc
Ganaxolone	Ganaxolone®	Neuroactive steroid	Ph3 Ph2	CDLK5 epilepsy PCDH19-related epilepsy Refractory status epilepticus	Drug resistant partial onset seizures Lennox-Gastaut Syndrome Smoking cessation PCDH19-related seizures Post-partem depression Fragile-X syndrome PTSD Infantile spasms	Marinus Pharmaceuticals
					Treatment-resistant depression as adjunct	
LYT-300		Neuroactive steroid	Preclinical	Not defined		Pure Tech Health
PF-06372865	CVL-865	α2/3/5 GABAkine	Ph2 Ph2 Ph2	Photosensitive epilepsy Chronic back pain GAD	Focal onset seizures Panic disorder (CO_2 model)	Cerevel Therapeutics
KRM-II-81		α2/3 GABAkine	IND enablement	Not defined		RespireRx Pharmaceuticals Inc

Other indications investigated were gleaned from the Clinical Trials Registry (https://clinicaltrials.gov/ct2/home).
FTXS: fragile-X associated tremor/ataxia disorder
GAD: generalized anxiety disorder
MDD: major depressive disorder
PTSD: post-traumatic stress disorder

A. Neuroactive steroids.

Three of the principal neuroactive steroids in clinical development are shown in **Fig. 6.** These GABAkines have potential for treating a host of neurological and psychiatric

disorders (Gasior et al., 1999; Miziak et al., 2020), and are currently in development primarily for epilepsy and depression. A role for neuroactive steroids in epilepsy has been postulated since the publication of early preclinical data (Belelli et al., 2019; Gasior et al., 1999; Reddy and Rogawski, 2012; Miziak et al., 2020).
Since neuroactive steroids generally have poor oral bioavailability, an oral prodrug form, LYT-300, is being developed (Puretech Health, n.d.).

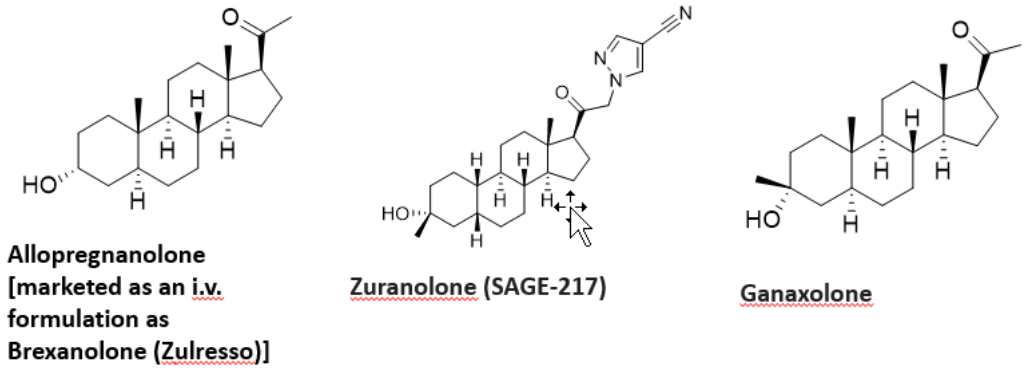

Figure 6. Chemical structures of the neuroactive steroid GABAkines in clinical development including an intravenous formulation of the endogenous steroid allopregnanolone, brexanolone Zulresso®), zuranolone, and ganaxolone. The structure of Sage-324 has not been disclosed. LYT-300 is an oral prodrug form of allopregnanolone whose structure has not been disclosed.

1. Allopregnanolone

a. Preclinical Pharmacology

Allopregnanolone is an endogenous GABAkine derived from progesterone. Preclinical studies have documented that allopregnanolone presents with the full spectrum of a GABA potentiation phenotype with anxiolytic-like, sedative-like, and anticonvulsant efficacy (Gasior et al., 1999; Reddy and Rogawski, 2010; Lévesque et al., 2017). Neuroactive steroids like allopregnanolone can prevent the development of seizure sensitization or kindling where diazepam is not effective (Gasior et al., 1999; Knutson et al., 2020). Brexanolone is a special formulation of allopregnanolone approved for the treatment of post-partum depression, and is a mixture of allopregnanolone and sulfobutylether- β-cyclodextrin (a solubilizing agent) (Scott, 2019). In the following paragraphs,brexanolone refers to this specific formulation, while all other formulations are referred to as allopregnanolone.

Work on the potential adjunctive treatment of seizure disorders with neuroactive steroids and conventional anticonvulsants began twenty years ago (Gasior et al., 2000).

b. Clinical. The current clinical application of brexanolone is for post-partem depression (Zorumski et al., 2019). An intravenous formulation of allopregnanolone constitutes the approved drug form marketed drug Zulresso for severe postpartum depression. The intravenous route by-passes the issue of allopregnanolone's poor oral bioavailability. Clinical studies have documented the efficacy of brexanolone (Frieder et al., 2019; Gerbasi et al., 2020; Kanes et al., 2017; Meltzer-Brody et al., 2018), the results of which have been summarized and are under discussion (Zheng et al., 2019; Patatanian and Nguyen, 2020; Kleinman and Schatzberg, 2021; Payne, 2021; Shukla et al., 2021). Although direct head-to-head comparisons with other agents have not been reported, indirect analyses of the data suggest that brexanolone produced larger changes in depression ratings as well as patient and clinician reports (Cooper et al., 2019). Recent post-hoc analyses of the three clinical trials for post-partem depression have indicated a rapid onset with intravenous treatment (about 60 h) and an antidepressant effect that endured to the 30-day evaluation period (Gerbasi et al., 2020). Analyses of these data also demonstrated the value of brexanolone treatment in improving health-related quality of life (Gerbasi et al., 2021).

Allopregnanolone has also been studied in patients with Fragile-X associated tremor/ataxia disorder. Wang et al. (2017) reported findings from six patients undergoing allopregnanolone infusions over 12 weeks. The patients showed improvements in executive functioning, episodic memory and learning; MRI data also suggested benefits in individual patients suggesting neuroprotective effects. Napoli et al. (2019) also reported beneficial effects of allopregnanolone treatment in patients with fragile X-associated tremor/ataxia syndrome when dosed for 12 weeks in an open-label study.

Another key area of clinical investigation has been in the area of status epilepticus and in particular super-refractory status epilepticus. Vaitkevicius et al (2017) provided the first findings that allopregnanolone could successfully block seizures in two patients (120-h infusion). Another study confirmed the efficacy and tolerability of allopregnanolone given as brexanolone infusion in 25 patients (Rosenthal et al., 2017).

The possible use of allopregnanolone in the treatment of Alzheimer's disease has also been considered. A tolerability study of allopregnanolone (formulated in a similar manner as brexanolone) was reported in 24 early Alzheimer's patients by Hernandez et al. (2020) where the safety and pharmacokinetic profiles suggested advancement into Phase-2 studies.

Brexanolone has also been in clinical investigation for other therapeutic indications (**Table 1**).

The safety and tolerability of brexanolone has been described now in multiple Phase-2 and3 clinical reports (see (Powell et al., 2020) for summary). Dizziness and somnolence are the most reported events but generally do not exceed 20%. Loss of consciousness is one of the biggest concerns that must be monitored and medically managed. Euphoria, especially at higher concentrations has been reported. The dosing protocol for brexanolone takes into account the major tolerability issues by dose escalation and patient monitoring.

c. LYT-300. An orally bioavailable prodrug form of allopregnanolone is being readied for Phase-1 studies in 2021. The compound avoids first-pass metabolism by the liver and was reported to achieve significant oral bioavailability of natural allopregnanolone in dog and non-human primate. LYT-300 is targeted for a range of neurological and psychiatric disorders as indicated by the sponsoring company, Puretech Health.

2. Zuranolone

a. Preclinical Pharmacology.

The synthesis of Sage-217 (zuranolone) was reported (compound 3) by Martinez-Botella et al. (2017). The bioisosteric pyrazole substitution on the ester function of an allopregnanolone backbone was made with the potential of increasing oral bioavailability and selectivity for GABAARs over other protein targets was demonstrated. The compound was described as a GABAkine with actions at synaptic and extrasynaptic sites and with oral bioavailability.

Data from the preclinical characterization of zuranolone were disclosed (Martinez Botella et al., 2017; Althaus et al., 2020). In electrophysiological studies, zuranolone amplified GABAAR currents at nine unique human recombinant receptor subtypes; synaptic (γ subunit-containing) as well as extrasynaptic (δ subunit-containing) currents were potentiated. Diazepam enhanced current was also amplified. Cell surface GABAAR trafficking was suggested by data from electrophysiologic studies in brain slices where sustained increases in current were induced. Oral activity was demonstrated in pharmacokinetic and efficacy assay readouts. Intraperitoneal dosing produced greater plasma levels (C_{max}) than oral dosing with good oral bioavailability (62% compared to 89% with i.p. administration). Brain to plasma ratios were 1.4 to 1.6 for i.p., and p.o. dosing respectively.

Tonic seizures induced by the $GABA_AR$ antagonist pentylenetetrazol (PTZ) were inhibited by zuranalone at a minimum effective dose of 1 mg/kg (trend at 0.3 mg/kg) in mice. Electroencephalographic studies in rats showed that oral administration augmented power in the β-frequency band and induced sleep at the higher doses of 3 and 20 mg/kg. These data provided another indicator of on-target engagement with oral dosing and a potential translatable biomarker for further compound development (Althaus et al., 2020).

Studies with another neuroactive steroid, SGE-516, have shown activity in animal models that detect compounds with anticonvulsant and mood-enhancing efficacy (Althaus et al., 2017; Hammond et al., 2017; Hawkins et al., 2017; Melón et al., 2018). The potential for neuroactive steroids to differentiate from other GABAkines is intriguing. This was first demonstrated in vivo with ganaxolone in a seizure kindling model where ganaxolone but not diazepam could prevent the development of kindling (Gasior et al., 1999) (see also similar data with KRM-II-81 in Knutson et al. (Knutson et al., 2020). In a model of Dravet Syndrome (Hawkins et al., 2017) and a model of post-partum depression (Melón et al., 2018), SGE-516 showed overlapping as well as distinct efficacy compared to clobazam. For example, in the Scn1a $^{+/-}$ mice that mirror some symptoms of Dravet

syndrome, SGE-516 increased long-term survival whereas clobazam was reported to be inactive against this biological endpoint (Hawkins et al., 2017). Although this differential pharmacology might be related to extrasynaptic activity of neuroactive steroids, a definitive explanation of differential mechanism has yet to be agreed upon.

a. Clinical Pharmacology

Phase-1 investigation of zuranolone was recently reported (Hoffmann et al., 2020). Single doses and dosing over 7 days were explored in healthy human volunteers as an oral solution. Pharmacokinetics and adverse events were monitored (see below) where the data encouraged advancement of the compound into a number of Phase-2 studies as well as pivotal studies in major depressive disorder and post-partum depression. As reported in preclinical studies, zuranolone was orally bioavailable and dose-proportional, with a terminal-phase half-life of 16-23 h and a t_{max} of about 1 h in humans.

Zuranolone was studied for potential efficacy in major depressive disorder (MDD) (Frieder et al., 2019). In a study by Gunduz-Bruce (2019), zuranolone was administered to MDD patients in a double-blind, placebo-controlled manner with a single oral dose of 30 mg. Fourteen days of daily treatment resulted in a statistically significant reduction in depression rating scores at day 15 compared to baseline. The reduction in symptoms across several measurement instruments was not associated with improvements in insomnia or anxiety (Gunduz-Bruce et al., 2019). Analysis showed that there was a favorable benefit/risk ratio in the administration of zuranolone in MDD patients (Arnaud et al., 2021). Unfortunately, the efficacy observed in the study by Gunduz-Bruce was not systematically replicated; zuranolone failed to meet its primary clinical endpoints in other studies (Pagliarulo, 2019).

Data from an open-label study of zuranolone (20-30 mg) in patients with Parkinson's disease was recently reported (Bullock et al., 2021). Patients were given zuranolone for 7 days while they were concurrently taking their dopaminergic medications. Zuranolone was safe and significantly reduced tremor in these patients.

In the dose-ranging study by Hoffmann et al. (2020), severe adverse events were observed after single oral dose at the level of 66 mg. These were reported as a change in mental status in two people, two people were unresponsive to external stimuli, and one showed marked somnolence. No loss of consciousness was observed. Sedation was the prominent side-effect reported. In the study with MDD patients, the most common adverse events in the drug group (45 patients) were headache, dizziness, nausea, and somnolence.

3. Sage-324

a. Preclinical Pharmacology.

Limited information has been made publicly available on this compound. It is reported by the company to be a neuroactive steroid GABAkine with oral bioavailability. In 2016,

the company reported that Sage-324 was a delta-preferring GABAkine in contrast to the balanced pharmacological profile of Sage-217 (Rosenthal and Metzer-Brody, 2016). The comparative in vitro pharmacology of these two compounds was reported as – Sage-324: α1β2γ2 (EC_e: 3000 nM); α4β3δ (EC_{50}: 273.4 nM) and Sage-217 α1β2γ2 (EC_{50}: 296.1 nM); α4β3δ (EC_{50}: 274.6 nM). These electrophysiological data show Sage-324 to be a α4β3δ–preferring GABAkine (ratio = 10.97) whereas Sage-217 is equipotent (ratio = 1.08). Brexanolone (Sage-547) was reported at the same time to have potencies of α1β2γ2 (EC_{50}: 184.6 nM); α4β3δ (EC_{50}: 80 nM) with a α4β3δ ratio of 2.31.

b. Clinical Pharmacology

Results of a study of Sage-324 in patients with essential tremor were disclosed in April 2021 (Ulrich, 2021). The bottom line on this clinical communication is that Sage-324 was well tolerated and efficacious in reducing essential tremor (36% reduction vs 21% with placebo at day 29 of treatment). The data also suggest the possible utility of this compound in other Phase-2 exploratory studies. Higher doses were explored and dose reduction was necessary in 62% of patients and discontinuations occurred in 38% of patients. The side-effects reported were about twice as high as in the placebo group - somnolence 68%; dizziness 38%; balance disorder 15%; diplopia 12%; dysarthria 12%; and gait impairments 12%.

4. Ganaxolone

a. Preclinical Pharmacology

Synergistic studies with tiagabine or midazolam in rodent seizure models have recently been reported (Chuang and Reddy, 2020). Using two mouse models, Reddy et al. (2019?)() found better efficacy of ganaxolone in females that they suggested is related to a greater abundance of extrasynaptic $GABA_A$Rs. A role for extrasynaptic $GABA_A$Rs in the anticonvulant actions of ganaxolone were further implicated with zinc blocking studies (Chuang and Reddy, 2019). Genotype differences in the efficacy of ganaxolone have also been reported in a mouse model of ethanol withdrawal-induced seizures (Nipper et al., 2019).

As with allopregnanolone, ganaxolone is under consideration for its potential beneficial efficacy in cases of nerve-agent-induced seizures (Reddy, 2019). Ganaxolone has also been studied as a possible intervention in Angelman syndrome where epilepsy is one of the developmental clinical features. Ciarlone et al. (2017) explored the effects of ganaxolone in the Ube3a-deficient mouse model where anxiolytic, anticonvulsant and motoric improving efficacy wereobserved. The impact of ganaxolone on status epilepticus was studied in rats by Saporito et al. (2019); ganaxolone exhibited prolonged plasma exposures and prolonged efficacy compared to allopregnanolone with both compounds showing profound sedative effects at higher doses.

Ganaxolone has also been explored in preclinical studies evaluating several neuropsychiatric disorders. In combination with clonazepam, ganaxolone was suggested to be supra-additive in augmenting anti-anxiety-like effects in rats (Gunter et al., 2016). Ganaxolone has shown preclinical efficacy in ameliorating behavioral deficits induced by

social isolation that in some cases are greater than that of fluoxetine (Locci et al., 2017). In a mouse model of autistic symptoms, ganaxolone showed enhancements in social behaviors (Kazdoba et al., 2016).

A role for ganaxolone in a host of psychiatric diseases has also gained recent preclinical scrutiny. Kazdoba et al. (2016) studied effects of ganaxolone in BTRB mice that model some aspects of autism spectrum disorder. From that study, they reported that ganaxolone improved some features of social approach and reciprocal social interactions in BTBR mice but did not significantly reduce repetitive self-grooming.

The abuse potential of ganaxolone was studied in primates where there were small differences in the drug-taking behavior of non-human primates compared to traditional benzodiazepine anxiolytics (Meng and Rowlett, 2016).

b. Clinical Pharmacology

Several clinical studies demonstrated the efficacy of ganaxolone in multiple epilepsy patient populations (Pieribone et al., 2007; Sperling et al., 2017; Yawno et al., 2017; Bialer et al., 2018). A Phase-3 study failed to meet its primary endpoint; however, analysis of a patient subgroup showed superiority over placebo (Bialer et al., 2018). Adverse events with ganaxolone have been mild and reversible and include somnolence, fatigue, dizziness, and headache (Bialer et al., 2018). Phase-2 and Phase-3 studies are ongoing and planned where ganaxolone is being developed for post-partum depression, pharmacoresistant status epilepticus, and several rare, treatment-resistant genetic epilepsies. Ganaxolone has been targeted as a potential treatment for pharmacoresistant status epilepticus (Zolkowska et al., 2018). Marinus Pharmaceuticals recently reported that ganaxolone successfully treated refractory status epilepticus in a Phase-2 trial (Meglio, 2019) and has progressed to Phase-3 clinical trial (Meglio, 2021). Recent reviews of the antiepileptic drug studies with ganaxolone are available (Miziak et al., 2020; Lattanzi et al., 2021), as well as its effects for childhood epilepsy (Perry, 2020), neonatal epilepsy (Yawno et al., 2017), and its neuroprotective properties (Thomas and Pang, 2020). Ganaxolone given at 1500 mg/day was reported to be an effective adjunct agent in the treatment of patients with partial-onset seizures (Sperling et al., 2017).

A good deal of work is also ongoing with ganaxolone in the area of orphan diseases. A negative trial was reported by Ligsay et al. (2017) in Fragile-X syndrome, although subgroup analysis suggested some potential benefits. Some recent reviews have been published for Lennox-Gastaut Syndrome (Strzelczyk and Schubert-Bast, 2021). A Phase-3 study is ongoing to evaluate ganaxolone in PCDH19-related epilepsy (Samanta, 2020).

An open-label study with ganaxolone as an add-on for depression in post-menopausal women that did not respond to ongoing conventional antidepressants was published (Dichtel et al., 2020) Ganaxolone was given b.i.d. in increasing doses over 8 weeks. Antidepressant efficacy was observed but with somnolence, fatigue, and dizziness. There were no significant effects on quality of life or sexual function. A review considering

treatments for post-partum depression has placed ganaxolone on the list (Frieder et al., 2019).

A commentary on the potential use of ganaxolone in patients with PTSD was published (Kawada, 2018) after the reported failed trial (Rasmusson et al., 2017).

B. PF-06372865 (darigabat, formerly CVL-865

The functional selectivity of PF-06372865 was determined in electrophysiological experiments on recombinant cell lines. PF-06372865 was determined to be selective for α2/3/5 relative to α1-associated $GABA_A$Rs (Nickolls et al., 2018; Owen et al., 2019 In rodent models, PF-06372865 blocked pentylenetetrazol and amygdala kindled seizures (Buhl et al., 2017) and dampened seizure activity in the GAERS rat, a genetic model of absence seizures (Duveau et al., 2018).

PF-06372865 was studied in healthy human volunteers and in patients where it was well-tolerated (Gurrell et al., 2018; Nickolls et al., 2018; Simen et al., 2019). PF-06372865 suppressed electrical activity in patients with photosensitive epilepsy (Gurrell et al., 2019) and has also demonstrated efficacy against multiple pain modalities in a Phase-1 clinical trial (van Amerongen et al., 2019). When tested in a larger Phase-2 trial for lower back pain, it did not achieve its primary efficacy end point of reduction in pain intensity and produced benzodiazepine-like side effects including sedation and memory impairment (Gurrell et al., 2018). In a clinical Phase-2 trial for anxiety, PF-06372865 failed for lack of efficacy and for induction of side effects (Simen et al., 2019). Lack of demonstrated efficacy might have been due to the relatively low estimated receptor occupancy (~50%) achieved with the maximal dose of 7.5 mg (Nickolls et al., 2018); however, the occurrence of somnolence, dizziness and memory impairment at this dose could preclude higher dose testing. Further development of PF-06372865 has been undertaken by Cerevel Therapeutics. The compound was renamed to darigabat (formerly CVL-865) and is in Phase-1 development as an anxiolytic (Zuiker et al., 2020) and Phase-2 as an antiepileptic (Dandurand, 2021).

In a small study of photosensitive epileptic patients, no severe side effects were reported with doses up to 52.5mg, however sedation and dizziness observed in half of the photosensitive epilepsy patients (Gurrell et al., 2019). Larger clinical trials for anxiety and chronic pain however reported adverse events
including dizziness, headache, somnolence, and cognitive impairment already at 7.5mg dose (Gurrell et al., 2018; Simen et al., 2019). A more recent Phase-1 study conducted by Cerevel dosed PF-06372865 twice daily for 14 days. The study reported no major side effects, with the most prevalent minor side effect being dizziness, which was in most cases of transient nature (Eides, 2019). A 42.5 mg dose of PF-06372865 was able to achieve >80% receptor occupancy without significant somnolence (Eides, 2019), whereas

benzodiazepines achieve 10% to 15% receptor occupancy with significant somnolence (Fujita et al., 1999).

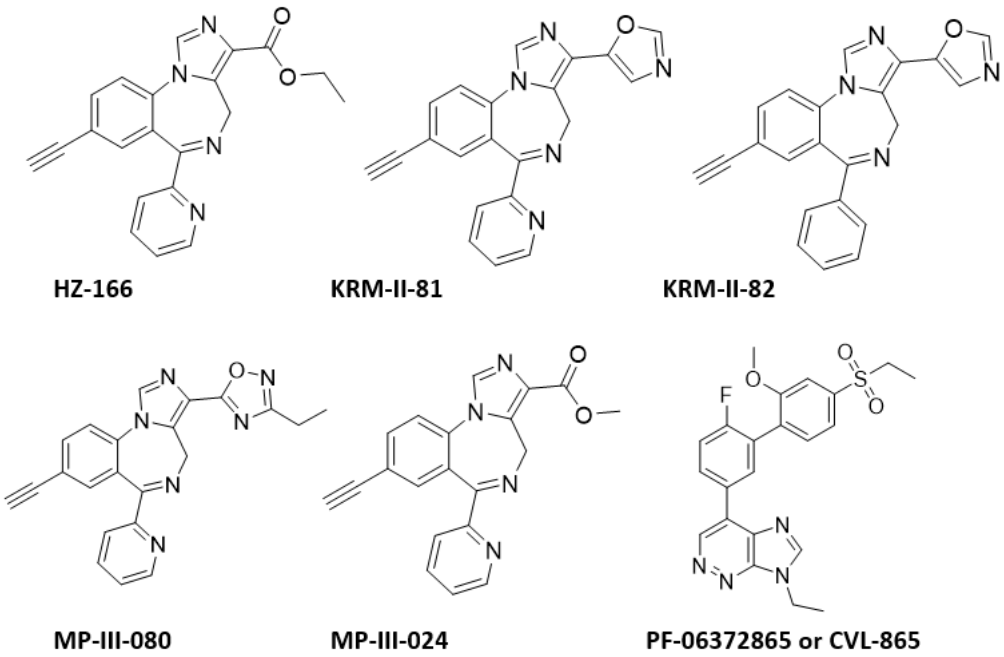

Fig. 7. Structures of the imidazodiazepine KRM-II-81 and structural analogs are shown. Also pictured is the structurally distinct compound PF-06372865. Both KRM-II-81 (α2/3-selective) and PF-06372865 (α2/3/5-selective), also known as CVL-865, are currently in development.

Table 2. Potency and efficacy of KRM-II-81 and the structural analog, MP-III-080, compared to diazepam and PF-06372865 (CVL-865).

Compound	$GABA_A$-α1		$GABA_A$-α2		$GABA_A$-α3		$GABA_A$-α4		$GABA_A$-α5		$GABA_A$-α6		Reference
	Potency (nM)	Efficacy (%)	Potency (nM)	Efficacy (%)	Potency (nM)	Efficacy (%)	Potency (nM)	Efficacy (%)	Potency (nM)	Efficacy (%)	Potency (nM)	Efficacy (%)	
KRM-II-81 (9)[2]	1730	115.6	101.9	252.24	60.9	262.22	>30000	97.425	192.6	114.1	>30000	98.775	Lewter et al., 2017
MP-III-80 (7)[2]	241.3	115.0	102.1	178.0	102.0	208.6	ND	ND	61.3	173.6	ND	ND	Witkin et al., 2017
Diazepam	18.1	256.24	17.6	286.54	19.6	272.92	>30000	100.28	11.1	211.18	>30000	100.42	Poe et al., 2016; Witkin et al., 2017
PF-06372865[3]	0.2	121.0	2.9	234.0	1.1	192.0	>19900	ND	18.0	191.0	>19900	ND	Owen et al., 2019

[1]Data are from different sources and sometimes from different methods as described in the references.
[2]Compound number in parentheses are compound designations in Poe et al. (2016) and in Witkin et al. (2017)
[3]Potency data are affinity values
ND: no data exist for these diazepam-insensitive $GABA_A$ receptor configurations

C. KRM-II-81

KRM-II-81 is another new GABAkine first disclosed as a non-sedating anxiolytic-like compound in 2016 from the laboratory of James M. Cook (Poe et al., 2016) (**Fig. 7**). KRM-II-81 is an imidazodiazepine GABAkine that preferentially activates α2/3-containing $GABA_A$Rs (**Table 2**). Preclinical data support the proposition that KRM-II-81 demonstrates reduced sedation, motor-impairing effects, tolerance development, and abuse liability compared to 1,4-benzodiazepine compounds. KRM-II-81 has also shown efficacy in animal models that are used to detect compounds that are anxiolytic, antidepressant, as well as those used in chronic and neuropathic pain states. KRM-II-81 has most extensively been studied as an anti-epileptic where it has shown greater potency and efficacy than diazepam in several animal models and greater efficacy in animal models of pharmaco-resistant epilepsy. In addition, KRM-II-81 has demonstrated *in situ* activity in human translational studies using brain tissue from treatment-resistant epileptic patients. The present section of this review summarizes key data in these experimental domains. No clinical data have been reported with KRM-II-81. It is currently under development by EndeavorRx, a business unit of RespireRx Pharmaceuticals Inc.

KRM-II-81 was first synthesized from HZ-166 by J. M. Cook and coworkers (Poe et al., 2016). HZ-166 (**Fig. 7**) was identified as an α2/3-preferring GABAkine that displayed reduced propensity for sedation and motor impairment compared to diazepam (Cook et al., 2009), and waswas active in models that detect anxiolytic drugs (Fischer et al., 2010), anticonvulsant compounds (Rivas et al., 2009), and was also active in models of pain in rodents (Di Lio et al., 2011). HZ-166 contains a metabolically-labile ester function that reduces its overall bioavailability (Poe et al., 2016), and KRM-II-81 was rationally designed to increase oral bioavailability and retain selectivity for α2/3-containing GABAARs by replacing the ethyl ester with an oxazole bioisostere. KRM-II-81 displayed good bioavailability after i.p. and oral administration in rats with exposure of plasma and brain. Both oral and intraperitoneal dosing in rats produced detectable unbound plasma levels of KRM-II-81 from 0.25-12h post dosing (Witkin et al., 2019a).

The ability of KRM-II-81 to amplify GABA signaling has been studied in electrophysiological systems. Studies have established KRM-II-81 as selective for potentiation of α2- and α3-containing $GABA_A$Rs with little amplification of α1-containing $GABA_A$Rs (**Table 2**). In contrast to KRM-II-81, diazepam also amplifies responses of GABA in recombinant cells containing α1- and α5-containing GABAARs (**Table 2**). Compared to PF-06372865 (α2/3/5- selective), KRM-II-81 is 9000 times less potent at α1 and 11 times less potent at α5 than PF-06372865. Thus, in addition to low efficacy at α1 and α 5-containing $GABA_A$Rs, KRM-II-81 requires higher concentrations to potentiate these 'off-target' $GABA_A$Rs.
When studied for its activity against a host of other receptor proteins, KRM-II-81 did not display any off-target liabilities (Poe et al., 2016).

Preclinical data have been reported in a broad range of seizure models using rodents and human epileptic brain tissue. Earlier studies had suggested that potentiation of α2/3-containing $GABA_A$Rs is sufficient for anticonvulsant effects (Rivas et al., 2009). KRM-II-81 displayed wide-ranging anticonvulsant activity (**Tables 3 and 4; Figs. 8 and 9**). In vivo, KRM-II-81 significantly suppressed convulsions induced by a range of chemical stimuli (**Table 3**). These effects were sometimes greater in efficacy than that achieved by diazepam although full dose-response curves have not always been studied (**Table 3**). The 6Hz mouse model is used to predict novel antiepileptic treatments (Barton et al., 2001) and potential agents for pharmaco-resistant epilepsies (Wilcox et al., 2013; Leclercq et al., 2014).

That α2-containing GABAARs are sufficient for anticonvulsant activity is evidenced by the finding that blockade of α1-containing GABAARs by β-CCT did not nullify the anticonvulsant effects of diazepam at doses that were sufficient to block its motor-impairing effects (Witkin et al., 2018). HZ-166 is an α2/α3-selective compound that has been reported to have anticonvulsant activity at non-motor-impairing doses in both mice (maximal electroshock, and PTZ) and rats (maximal electroshock, PTZ, and hippocampal kindling) (Rivas et al., 2009).

KRM-II-81 also displays anticonvulsant activity in seizure sensitization models, pharmacoresistant epilepsy models, and in human epileptic tissue (**Table 4; Fig. 9**). The occurrence of seizures increases the future probability of seizure occurrence, an effect known as seizure sensitization or seizure kindling (Goddard et al., 1969). Seizure kindling can be modeled in rodents by the delivery of daily subthreshold levels of convulsant stimulation (e.g., chemical or electric). KRM-II-81 blocked fully kindled seizures induced by electrical corneal stimulation, electrical stimulation of the basolateral amygdala, and by the chemoconvulsants, pentylenetetrazol and cocaine (**Table 4**).

Pentylenetetrazol-induced kindling is shown as an example in **figure 9**. Every other day dosing with pentylenetetrazol (45 mg/kg) resulted in increases in seizure prevalence over days. On day 10, either vehicle, KRM-II-81 (30 mg/kg), or diazepam (1 mg/kg) was given prior to pentylenetetrazol. KRM-II-81 and diazepam completely suppressed convulsions in these <u>fully-kindled mice</u> (**Fig. 9A**). KRM-II-81 and diazepam also blocked convulsions when given every day in the presence of the kindling dose of pentylenetetrazol (blockade of the <u>expression of kindling</u>) (**Fig. 9B**). Daily treatment with KRM-II-81 + pentylenetetrazol also prevented the <u>development of seizure kindling</u>; mice tested on day 10 with pentylenetetrazol in the absence of KRM-II-81 exhibited a significant reduction in seizure prevalence compared to the mice given vehicle + pentylenetrazol prior to the test session. Diazepam was considerably less effective than KRM-II-81in reducing kindling development (**Fig. 9C**).

c. Pharmacoresistant models. Epilepsy can become resistant to antiepileptic drug therapy (Franco et al., 2014). In a mesial temporal lobe epilepsy model in mice, KRM-II-81 was an effective anticonvulsant under conditions in which standard of care antiepelipetics such as lamotrigine and valproic acid were not. In this model, mice develop enduring epileptic events that are characteristic of temporal lobe epilepsy in patients (Bouilleret et al., 1999; Riban et al., 2002). Mice with kainate-induced mesial temporal lobe seizures exhibited spontaneous recurrent hippocampal paroxysmal discharges. These discharges occurred from 30-60 times per hour and lasted about 15-20 sec. Baseline levels of spontaneous discharges were 16.8 $\pm$ 2.5. In the presence of 15 mg/kg KRM-II-81 (p.o., 2h prior), there was a significant reduction in spontaneous discharges to a mean of 5.5 $\pm$ 1.4 discharges.

In the lamotrigine-insensitive model of pharmacoresistant epielpsy (Srivastava et al., 2013; Wilcox et al., 2013), KRM-II-81 protected rats from convulsions with an ED_{50} of 19.2 mg/kg. KRM-II-81 also reduced the severity of electrically-driven seizures with a minimal effective dose of 5 mg/kg (**Fig. 8**).

Another model uses multiple doses of kainate to create a chronic state of epilepsy that is not responsive to a host of antiepilptic drugs. Under these conditions, kainate induces a long-term sequalae of focal and generalized seizures. This model enables the monitoring of spontaneous recurrent seizures and clinically-relevant measures of epilepsy for antiepileptic drug differentiation (West et al., 2018). In this model, KRM-II-81 (20 mg/kg, t.i.d) decreased the seizure burden (number of seizure events/day) and increased the percentage of rats that remained seizure free (**Fig. 8**).

The mechanisms for pharmaco-resistance are likely multifaceted; nonetheless, tolerance development has been suggested to play a major role (Löscher and Schmidt, 2006). KRM-II-81 did not produce tolerance, over five days of dosing, to the suppression of clonic convulsions in mice induced by the chemoconvulant pentylenetetrazol; diazepam had comparable effects (Witkin et al., 2018). In the kainate-induced chronic epilepsy model, KRM-II-81 given daily for five days did not exhibit decreases in anticonvulsant efficacy (**Fig. 8**). Similar findings were reported by Rivas et al. (2009) with HZ-166. Longer term dosing studies are required to determine whether KRM-II-81 has a reduced propensity to produce tolerance as suggested by the data in pain models (**Fig. 10**) and as predicted from point mutation studies implicating α2-containing GABAARs as a target for the relief of tolerance development (Ralvenius et al., 2015).

Table 3. Effects of KRM-II-81 in seizure models: Neuronal culture, chemical seizure provocation models, and electrical seizure provocation models. Efficacy superior to the efficacy of diazepam is highlighted in green.

Model System	Species	Efficacy	Reference
NEURONAL CULTURE			
Dissociated cortical neurons	Rat	ND	Witkin et al., 2018

CHEMICAL SEIZURE PROVOCATION MODELS			
Pentylenetetrazol – clonic seizures	Rat	= Diazepam	Witkin et al., 2018
Pentylenetetrazol – clonic seizures	Mouse	= Diazepam	Knutson et al., 2020
Pentylenetetrazol – tonic seizures	Mouse	= Diazepam	Knutson et al., 2020
Pentylenetetrazol – lethality	Mouse	= Diazepam	Knutson et al., 2020
Pentylenetetrazol – seizure threshold	Rat	> Diazepam	Witkin et al., 2018
Cocaine – clonic seizures	Mouse	> Diazepam	Knutson et al., 2020
4-Aminopyridine – clonic seizures	Mouse	> Diazepam	Knutson et al., 2020
4-Aminopyridine – tonic seizures	Mouse	> Diazepam	Knutson et al., 2020
4-Aminopyridine – lethality	Mouse	= Diazepam	Knutson et al., 2020
NMDA – clonic seizures	Mouse	> Diazepam	Knutson et al., 2020
NMDA – lethality	Mouse	> Diazepam	Knutson et al., 2020
Picrotoxin – clonic seizures	Mouse	= Diazepam	Knutson et al., 2020
Picrotoxin – tonic seizures	Mouse	> Diazepam	Knutson et al., 2020
Picrotoxin – lethality	Mouse	> Diazepam	Knutson et al., 2020
Strychnine – clonic seizures	Mouse	> Diazepam	Knutson et al., 2020
Strychnine – tonic seizures	Mouse	> Diazepam	Knutson et al., 2020
Strychnine – lethality	Mouse	> Diazepam	Knutson et al., 2020
Pilocarpine – clonic seizures	Mouse	= Diazepam	Knutson et al., 2020
Pilocarpine – lethality	Mouse	= Diazepam	Knutson et al., 2020
ELECTRICAL SEIZURE PROVOCATION MODELS			
6Hz stimulation – 44mA	Mouse	ND	Witkin et al., 2018
Electroconvulsive Shock	Mouse	= Diazepam	Witkin et al., 2018

ND: no data

Table 4. Effects of KRM-II-81 in seizure models: Seizure sensitization models, pharmacoresistant models, and human epileptic tissue. Efficacy superior to the efficacy of diazepam is highlighted in green.

Model System	Species	Efficacy	Reference
SEIZURE SENSITIZATION			
Corneal kindling	Mouse	>Tpm	Witkin et al., 2020
Amygdala kindling-ADT	Rat	> Diazepam	Witkin et al., 2018
Amygdala kindling-ADD	Rat	= Diazepam	Witkin et al., 2018
Amygdala kindling-Seizure Severity	Rat	= Diazepam	Witkin et al., 2018
Pentylenetetrazol kindling – Fully kindled	Mouse	= Diazepam	Knutson et al., 2020
Pentylenetetrazol kindling - Expression	Mouse	= Diazepam	Knutson et al., 2020
Pentylenetetrazol kindling - Development	Mouse	> Diazepam	Knutson et al., 2020
Cocaine kindling-– Fully kindled	Mouse	> Diazepam	Knutson et al., 2020
Cocaine kindling- Expression	Mouse	> Diazepam	Knutson et al., 2020

Cocaine kindling- Development	Mouse	> Diazepam	Knutson et al., 2020
PHARMACORESISTANT MODELS			
Mesial temporal lobe epilepsy	Mouse	>Ltg, Val	Witkin et al., 2020
Ltg-insensitive kindling	Rat	>Ltg, Tpm	Witkin et al., 2020
Kainate-induced chronic epilepsy	Rat	>Ltg, Lev	Witkin et al., 2020
HUMAN EPILEPTIC TISSUE			
Picrotoxin stimulation	Human	Active	Witkin et al., 2018
4-Aminopyridine stimulation	Human	Active	Witkin et al., 2018
4-Aminopyridine stimulation	Human	Active	Unpublished

Lev: leviteracetam; Ltg: lamotrigine; Tpm: topiramate

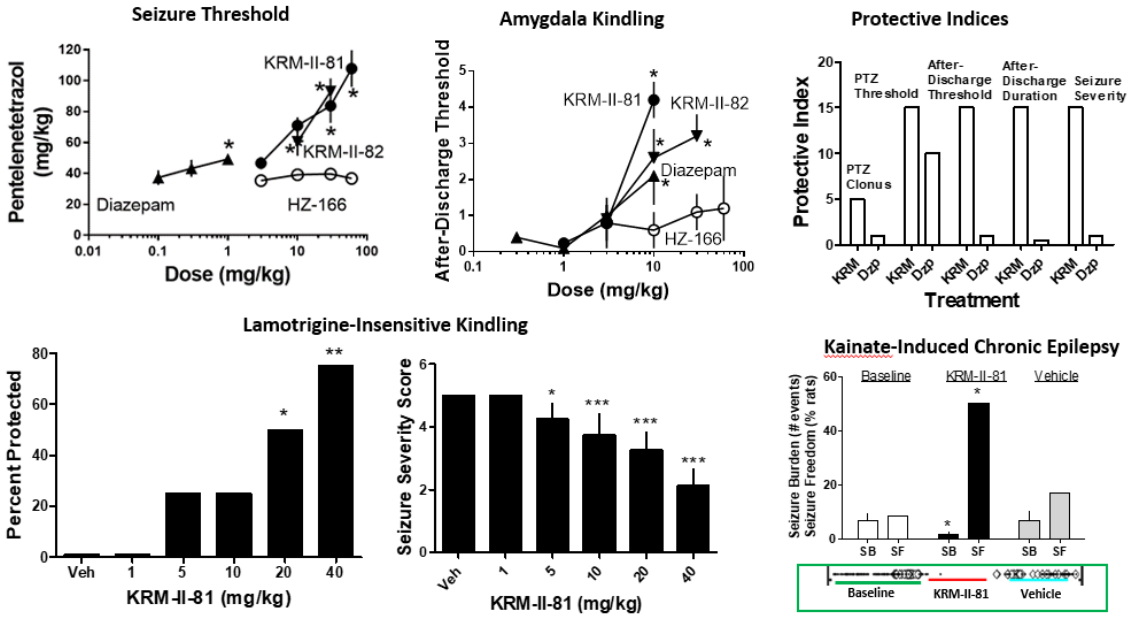

Figure 8. Effects of KRM-II-81 and comparator compounds in some rodent seizure models. **Seizure threshold.** Pentylenetetrazol seizure thresholds (dose required to induce seizure) were significantly increased by KRM-II-81 and KRM-II-82 but not by HZ-166. Diazepam is shown as a comparator. Each point represents the mean ± SEM effect in groups of 8 rats. * $p<0.05$ compared to vehicle control (35.1 ± 1.2 mg/kg). **Amygdala kindling.** Comparative effects of HZ-166, KRM-II-81, KRM-II-82, and diazepam in rats that were seizure kindled to daily electrical stimulations of the basolateral amygdala. Each point represents the mean ± SEM effect (n=8). * $p<0.05$ compared to vehicle control. **Protective indicies.** Ratios of doses inducing motor impairment / doses inducing anticonvulsant efficacy. **Lamotrigine-insensitive kindling.** KRM-II-81 increased the percentage of rats protected and decreased the seizure severity scores in rats (n=8) kindled in the presence of lamotrigine. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to vehicle control (veh). **Kainate-induced chronic epilepsy.** Effects of KRM (20 mg/kg, i.p., t.i.d.) on seizure burden and seizure freedom in a group of 12 rats in a cross-over design. SB: seizure burden; SF: seizure freedom. * $p< 0.05$ compared to vehicle control. Data from the top panels are from (Witkin et al., 2018) with permission of the publisher. Figures on the bottom panels are replotted from data presented in (Witkin et al., 2020).

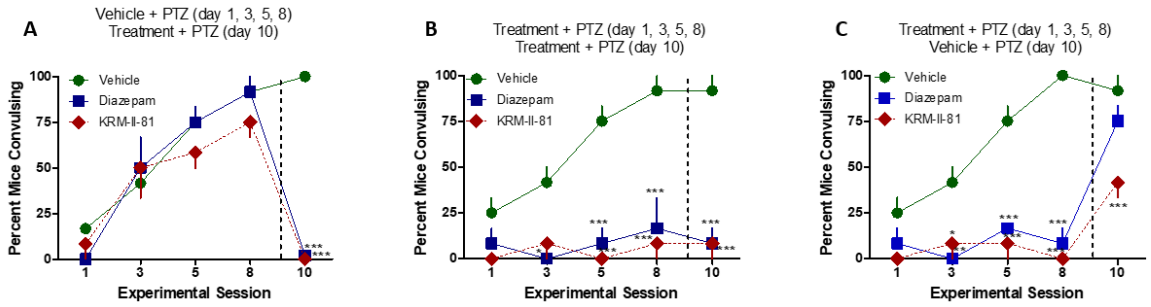

Figure 9. Comparative effects of KRM-II-81 and diazepam on pentylenetetrazol (PTZ)-induced seizure kindling in mice. **A.** Fully developed PTZ kindled seizures. **B**. Expression of PTZ kindling. **C.** Development of PTZ kindling. Each point represents the mean + S.E.M. of 6 mice each in two separate experiments. *p<0.05; ***p<0.001 compared to their respective vehicle control for each experimental session. Data presented are the percentage of mice exhibiting convulsions and are from Knutson et al. (2020) with permission of the publisher.

d. Post-traumatic epilepsy

Traumatic brain injury engenders long-term negative health outcomes including posttraumatic epilepsy that is associated with affective, neurocognitive, and psychosocial disruption of life (Semple et al., 2019) and for which there are no effective treatments (Temkin, 2009; Wat et al., 2019). Ping and Jin (2016) described a sequelae of events post TBI using a mouse model - an early quiescence phase of neuronal activity is followed by sustained hyperactivity of cortical neurons at the time when the mice are at increased risk to develop posttraumatic epilepsy (Bolkvadze and Pitkänen, 2012).

Effects of KRM-II-81 on inhibiting cortical network activity was studied using an *in vivo* two-photon imaging technique in which neuronal activity in cortical layer II/III pyramidal neurons was assessed in mice with traumatic brain injury. In this work, the dramatic increases in fluorescence, measuring the intensity of spiking activity of individual neurons and fraction of active neurons, was observed 3 months after brain injury and imaging over time of these mice illustrated the activity increases. Both the mean integrated fluorescence and fraction of active neurons were markedly decreased after KRM-II-81 (Witkin et al., 2020). The possibility that KRM-II-81 might also positively reduce the probability of post-traumatic epilepsy is raised by these findings.

e. Human Epileptic Tissue. In order to further establish translatability of the anticonvulsant effects of KRM-II-81 to patients, human epileptic brain tissue has been used. Recordings were made from freshly transected cortical tissue slices from the brains of juvenile epileptic patients that were refractory to antiepileptic medications.

KRM-II-81 decreased picrotoxin-induced and 4-aminopyridine-induced increases in cortical firing rates (Witkin et al., 2018).

4. Antinociceptive activity.

a. GABA and Pain. Pain pathways rely heavily on GABAAR-driven inhibitory neurotransmission (Hammond and Drower, 1984; Dirig and Yaksh, 1995; Enna and McCarson, 2006; Zeilhofer et al., 2015; Etlin et al., 2016). A nice illustration was presented over 25 years ago. Inflammatory pain induced by formalin injection in rat paws was evidenced by hyperalgesic responses to tactile stimulation. Intrathecal injection of the direct-acting agonist muscimol produced antihyperalgesic efficacy (phase II of formalin assay) but also produced some sedative-like effects (phase I) (**Fig. 10A**). That these effects of muscimol were due to stimulation of GABAARs was confirmed through the use of the GABAAR antagonist bicuculline (**Fig. P1A**). More recent studies have shown that intrathecal muscimol can also reduce neuropathic pain endangered by spinal cord injury in rats (Hosseini et al., 2014).

b. α2-Containing GABAARs and Pain. Although GABA is known to be an integral biological mediator of pain, GABAkines are generally not used to control pain (Chou et al., 2017) although prescriptions for pain patients occurs (Wright, 2020). The sedative properties of benzodiazepines have been proposed to interfere with the attainment of sufficiently high doses to produce analgesic benefit (McKernan et al., 2000; Knabl et al., 2009; Munro et al., 2009; Atack, 2010; Ralvenius et al., 2015). A potential role for α1-containing GABAARs in the mitigation of the antinociceptive effects of diazepam has been demonstrated in point mutation studies. **Figure 10B** shows that diazepam produces dose-dependent anti-nociceptive effects in the formalin test in mice only if α1-containing GABAARs are negated. That α2-containing GABAARs are responsible for this emergent anti-nociceptive activity was provided by the finding that the α2-selective GABAkine, HZ-166, was analgesic in the formalin assay in normal mice but was absent in mice where the activation of α2-containing GABAARs was negated by point mutation (**Fig. 10C**). Point mutation studies have continued to confirm a key role for α2-containing GABAARs in the regulation of pain at a spinal level (Paul et al., 2014; Tudeau et al., 2020). That α2-preferrning GABAAR GABAkines can produce anti-nociceptive activity is summarized in **Table 5**.

Table 5. Data relating α2/3-containing $GABA_A$ receptors to pain.

STUDY	EVIDENCE	REFERENCE
Genetic Models	Sedative, amnesic and anticonvulsant actions of diazepam are reduced in α1(H101R) mice*	(Rudolph et al., 1999; McKernan et al., 2000)
	Analgesic effect of spinal diazepam is reduced in α2(H101R) and to lesser extent in α3(H126R) mice*	(Knabl et al., 2008)
	Analgesia by systemic diazepam is unmasked in α1(H101R) mice*	(Knabl et al., 2009)
	Intact α2 GABAAR is sufficient for mediation of diazepam mediated antihyperalgesia**	(Ralvenius et al., 2015)
	Intact α1 GABAAR is sufficient for mediation of diazepam mediated sedation**	(Ralvenius et al., 2015)
Preclinical Models	L-838417 is analgesic in inflammatory and neuropathic pain	(Knabl et al., 2008; Nickolls et al., 2011; Hofmann et al., 2012; Lorenzo et al., 2020)
	NS11394 is analgesic in inflammatory and neuropathic pain	(Munro et al., 2008, 2009)
	NS16085 is analgesic in inflammatory and neuropathic pain	(de Lucas et al., 2015)
	TPA023 is analgesic in inflammatory and neuropathic pain	(Nickolls et al., 2011)
	TPA023B blocks itch mice that is α2/3-dependent	(Ralvenius et al., 2018)
	PF-06372865 is analgesic in neuropathic pain	(Owen et al., 2019)
	HZ-166 is analgesic in inflammatory and neuropathic pain without sedation or tolerance	(Di Lio et al., 2011)
	Antihyperalgesia by HZ166 is reduced when α2-GABAARs were benzodiazepine insensitive	(Paul et al., 2014; Ralvenius et al., 2015)
	NDMC (α2 preferring clobazam metabolite) has improved therapeutic window for antihyperalgesia	(Ralvenius et al., 2016)
Human Studies	Clobazam decreased the area of secondary hyperalgesia in human volunteers	(Vuilleumier et al., 2013; Besson et al., 2015)
	NDMC failed to reduce hyperalgesia in human volunteers	(Matthey et al., 2020)
	Hypnotic effect of zolpidem is driven by preference for α1-GABAARs	(Wafford et al., 1993; Hevers and Lüddens, 1998)
	PF-06372865 displayed PD biomarker engagement in Phase-1 clinical study	(Nickolls et al., 2018)
	PF-06372865 suppressed evoked pain (electrical, pressure, heat, cold, inflammatory)	(van Amerongen et al., 2019)
	PF-06372865 failed to demonstrate efficacy in Phase-2 for chronic low back pain (no sedation)	(Gurrell et al., 2018)

NDMC - N-desmethyl-clobazam

* Studies are using point mutated mice in which α-subunits were rendered diazepam insensitive (α1(H101R), α2(H101R), α3(H126R) and α5(H105R)).

** Studies using triple mutant mice in which only one α-subunit is diazepam sensitive while other α-subunits were diazepam insensitive.

The association of diazepam with motor impairment and lack of antinociceptive efficacy is shown also in **figure 10D** where diazepam, given up to motor-impacting doses (**Fig. 10D insert**), was not effective in the formalin assay in rats. In contrast, KRM-II-81 suppressed pain and did not suppress locomotor activity of rats up to doses of 100 mg/kg (**Fig. 10E**). Taken as a whole, the data are consistent with the hypothesis that while potentiation of GABAergic neurotransmission induces antinociceptive efficacy, this outcome requires doses of α1-enhancing GABAkines that are above their sedative and motor-impacting doses. Furthermore, the data with HZ-166 and KRM-II-81 show that selective amplification of GABA signaling through α2/3-containing GABAARs is sufficient for the transduction of antinociception.

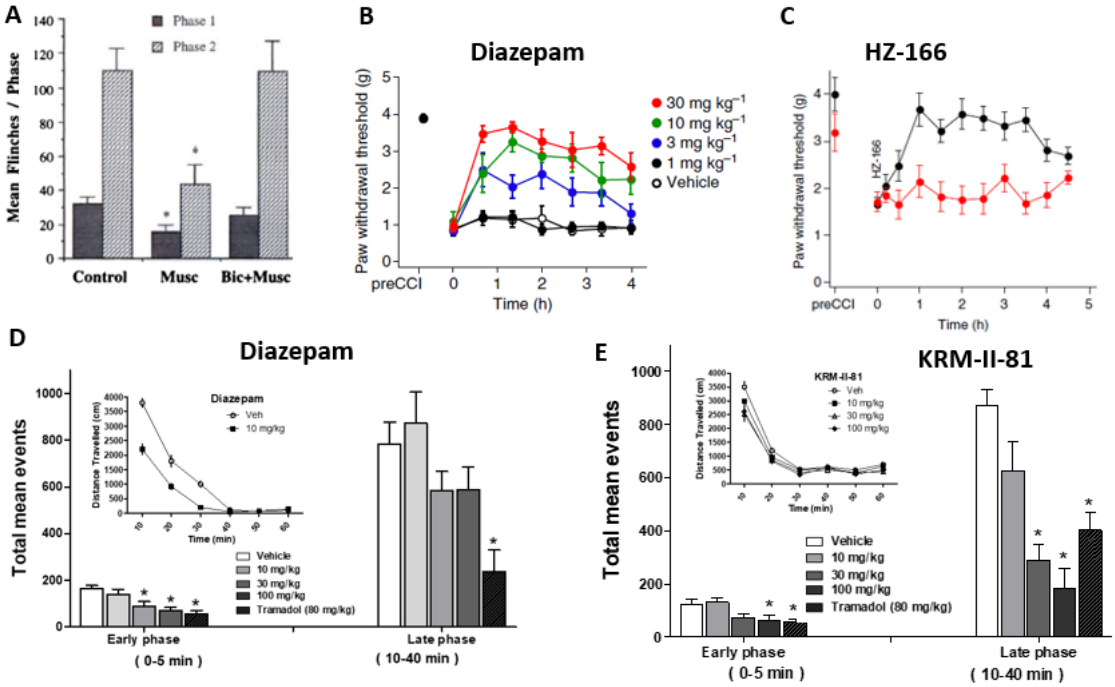

Figure 10. Effects of compounds in various pain models in rodents. **A.** Muscimol decreased paw flinching in a rat formalin assay; this effect was prevented by bicuculine. Each bar represents the mean + SE (n=8). *p<0.05 compared to vehicle. Data are from Dirig and Yaksh (1995) by permission of the publisher. **B.** Diazepam increased paw withdrawal thresholds in mice with α1point mutations that were rendered hyperalgesic from chronic constriction injury. Data are means ± SEM (n=6-7). Diazepam was not effective in wild-type mice (data not shown). Data are from Ralvenius et al. (2015) with permission of the publisher under the Creative Commons license, http://creativecommons.org/licenses/by/4.0/. **C.** HZ-166 (16 mg/kg, i.p.) increased paw withdrawal thresholds in wild-type mice (black), an effect negated in mice with α2 point mutations (red). Data are means ± SEM (n=7-8). Data are from Ralvenius et al. (2015) with permission of the publisher under the Creative Commons license, http://creativecommons.org/licenses/by/4.0/. **D.** Effects of diazepam on paw withdrawal events

in rats after formalin injection compared to tramadol. The figure inset shows locomotor activity. Data are means + SEM in 6 (locomotion) or 8 rats. *p<0.05 compared to vehicle control. Data are from Witkin et al. (2019)) with permission of the publisher. **E.** Effects of KRM-II-81 on paw withdrawal events in rats after formalin injection compared to tramadol. The figure inset shows locomotor activity. Data are means + SEM in 8 rats. *p<0.05 compared to vehicle control. Data are from Witkin et al. (2019a) with permission of the publisher.

The potential for this mechanism to serve to reduce opioid burden was reported by Rahman et al. (2021). The KRM-II-81 ester analog, MP-III-024, was studied as an adjunct in rat models of mechanical and thermal hyperalgesia. Given alone, MP-III-024 was active in the mechanical model whereas morphine was active in both. When dosed together, MP-III-024 engendered synergistic effects.

c. Chronic Neuropathic Pain. KRM-II-81 and structural analogs have been studied in in both mice and rats in acute and chronic pain models where efficacy was first reported by Lewter (Lewter et al., 2017, 2018) (see **Table 6**).

Table 6. Effects of KRM-II-81 and structural analogs in rodent pain models

Compound	Pain model	Species	Compartors	References
KRM-II-81	Acetic and lactic-acid-induced writhing, nesting and locomotion	ICR mice	Morphine	Lewter et al. (2017)
KRM-II-18B	Acetic and lactic-acid-induced writhing, nesting and locomotion	ICR mice	Morphine	Lewter et al. (2017)
KRM-II-81	Lactic-acid and ICSS behavior	Sprague Dawley rats	Ketorolac and diazepam	Moerke et al. (2019)
MP-III-024	Zymosin A-induced mechanical hyperalgesia	C57BL/6 mice	Gabapentin	Fischer et al., 2017
KRM-II-81	Formalin-induced tactile hyperalgesia	Sprague-Dawley rats	Tramadol and diazepam	(Witkin et al. (2019)
KRM-II-81	L5/6 nerve ligation – induced tactile hyperalgesia	Sprague-Dawley rats	Gabapentin	(Witkin et al. (2019)
KRM-II-81	L5/6 nerve ligation – senitization training - induced tactile hyperalgesia	Sprague-Dawley rats	Gabapentin	(Witkin et al. (2019)
KRM-II-81	Chemotherapy-induced thermal hyperalgesia	C57BL/6 mice	Gabapentin	Biggerstaff et al. (2020)
KRM-II-81	Chemotherapy-induced tactile hyperalgesia	C57BL/6 mice	Gabapentin	Biggerstaff et al. (2020)
HZ-166	Zymosin A-induced mechanical hyperalgesia	C57BL/6 mice	Gabapentin	Di Lio et al. (2011)
HZ-166	Chronic constriction injury	C57BL/6 mice	Gabapentin	Di Lio et al. (2011)
HZ-166	Inflammotory bladder pain	Neonatal Sprague-Dawley rats	No	Kannampalli et al. (2017)

Two examples of the effects of KRM-II-81 in chronic pain states are illustrated in **figure 11**. In a study using rats, L5/6 nerve ligation markedly produced long term increases in tactile allodynia. KRM-II-81 increased these pain thresholds close to baseline levels with potency and efficacy greater at some time periods greater than gabapentin, the standard-of-care agent (**Fig. 11A**). Unbound plasma levels of KRM-II-81 were positively associated with antinociceptive efficacy (Witkin et al., 2019a).

Chronic neuropathic pain induced by the chemotherapeutic agent was studied in mice. KRM-II-81 and MP-III-080 produced dose-dependent reversal of both mechanical and thermal allodynia (Biggerstaff et al., 2020). All three drugs also fully reversed the chemotherapy-induced allodynia when dosed from day 18 to day 40 (**Fig. 11B**). However, tolerance developed to the effects of gabapentin but not to KRM-II-81 or MP-III-080 (**Fig. 11B**).

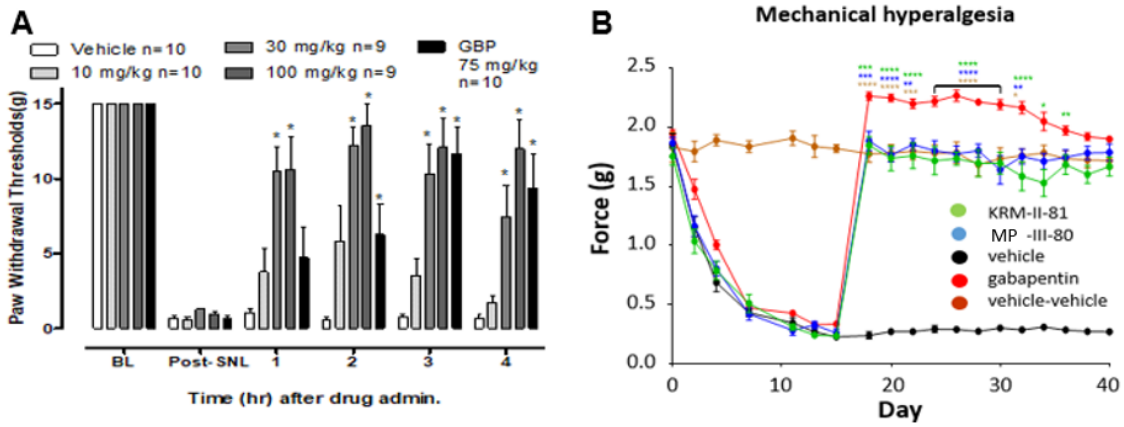

Fig. 11. A. KRM-II-81 (p.o., 60 min prior) decreased the hyperalgesic responses of rats with chronic spinal nerve ligation (L5/6)-induced chronic pain. KRM-II-81 (10 - 30 mg/kg) or gabapentin (75 mg/kg) were dosed orally. BL: baseline paw withdrawal latencies prior to spinal nerve ligation (SNL). Post-SNL: paw withdrawal latencies after spinal nerve ligation. *p<0.05 compared to vehicle control. Data are from Witkin et al. (2019a) with permission of the publisher. **B.** Effects of daily KRM-II-81, MP-III-080, or gabapentin administration (all given i.p.) on chronic neuropathic pain (Day 18-40) induced by paclitaxel in mice (n=6-7). * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. Data are from Biggerstaff et al. (2020) with permission of the publisher.

d. Tolerance. Tolerance to the antinociceptive effects of pain medications creates serious health risks both by placing the patient in undue pain and increased risk of medication dependence and lethality (Kalant et al., 1971; Huxtable et al., 2011). In a study of CFA-induced inflammatory pain, daily dosing with midazolam induced a 1.8- and 2.9-fold rightward shift in the analgesic dose-response curve after 3 and 7 days of dosing (b.i.d.), respectively. No tolerance was seen with KRM-II-81 after 3, 7, or 11 days of subchronic dosing (Lewter, 2019). Similar findings were observed in a neuropathic pain preparation with chronic constrictive nerve injury; midazolam produced a 1.4- and 2.7-fold shift to the right in the dose-effect curve after 3 and 7 days of repeat dosing (b.i.d.), respectively. No tolerance to the antinociceptive effects of KRM-II-81 was observed after 3, 7, or 11 days of subchronic dosing (Lewter, 2019).

The antinociceptive efficacy of KRM-II-81 and MP-III-080 was also enduring over a 22-day period of subchronic dosing (Biggerstaff et al., 2020). The lack of tolerance by KRM-II-81 and MP-III-080 is consistent with data on HZ-166 where no tolerance was observed after 9 days in mouse models of neuropathic and inflammatory pain (Di Lio et al., 2011). Additional studies are needed to fully appreciate the limits of the absence of tolerance development (drug exposures and durations as well as different dependent measures) and the mechanisms associated with this phenomenon.

5. Anxiolytic and antidepressant activity.

Reduced sedation with directed on-target therapeutic activity has been a long-standing goal for anxiolytic drug discovery. A host of compounds were synthesized and tested in animal models with this goal in mind (Gee and Yamamura, 1982; Patel et al., 1982; Griebel et al., 1999; Atack, 2008). As a result, many compounds were advanced into clinical development. The dependence-producing properties and abuse liability of 1,4-benzodiazepine anxiolytics has made the selective serotonin reuptake inhibitor antidepressant/anxiolytics a first line therapy for anxiety (Baldwin et al., 2005).

However, these anxiolytic antidepressants are not acutely effective in treating anxiety as are the GABAkines and have their own line of side effects (see discussion below).

KRM-II-81 and analogs produced anxiolytic-like effects in rodents at doses that do not produce sedation or motor-impairment. This was reported in a marble-burying assay in mice (Poe et al., 2016; Knutson et al., 2020) and in a Vogel-conflict study in rats (Poe et al., 2016; Witkin et al., 2017).

GABAkines like diazepam are not generally prescribed for the treatment of major depressive disorder. They do find use in anxious depression (Benasi et al., 2018) and in other forms of depression with less defined outcome (van Marwijk et al., 2012). They are sometimes used for augmenting antidepressant response but are not the top tier agents used for augmentation (Ogawa et al., 2019; Dold et al., 2020). However, neuroactive steroids that allosterically potentiate GABAergic neurotransmission have received recent attention for their potential impact on depression where brexanolone is an approved therapy for post-partum depression (**Section VI A1**).

In preclinical models, benzodiazepines are generally not detected as antidepressants in preclinical screens (Kostowski et al., 1986; Nagatani et al., 1987). However, antidepressant-like effects have been detected at some doses in the forced-swim test in some studies with alprazolam (Flugy et al., 1992; El Zahaf and Elhwuegi, 2014), midazolam (Qiu et al., 2015), and neuroactive steroids (Khisti et al., 2000). Effects in antidepressant-detecting models are dose-dependent with higher doses generally increasing rather than decreasing immobility times (El Zahaf and Elhwuegi, 2014).

KRM-II-81 and two structural analogs have been evaluated for their potential antidepressant-like effects in mice. KRM-II-81 exhibited antidepressant-like effects in mice when studied in the forced-swim but not the tail-suspension test (Methuku et al., 2018). In contrast to KRM-II-81, diazepam was not active in the forced-swim test (Methuku et al., 2018). Attenuating the influence of diazepam on α1-containing GABAARs with β-CCT, a selective antagonist of this subpopulation of GABAARs (Huang et al., 2000), blocked the motor-impairing effects of diazepam and enabled the antidepressant-like effects of KRM-II-81 to be unveiled (Methuku et al., 2018).

Clinical data with eszopiclone might also lend some support to an α2/3 protein hypothesis of antidepressant activity. This molecule has activity at α2/3-containing GABAARs and augments the antidepressant effects of the selective serotonin reuptake inhibitors, fluoxetine or escitalopram (Fava et al., 2006, 2011b; Krystal et al., 2007), an effect not observed with the α1-selective PAM, zolpidem (Fava et al., 2011a).

7. Side-effect profile.

a. CNS and motor impairment. On multiple measures of sedation and motor impairment, KRM-II-81 was less impacting than 1,4-benzodiazepines like diazepam (Poe et al., 2016; Lewter et al., 2017; Witkin et al., 2018) **Fig. 8; Fig. 10D**).

The data for KRM-II-81 is reminiscent of the findings with the clinically-validated non-sedating GABAkine ocinaplon (Lippa et al., 2005).

Given the reduced motoric impact of KRM-II-81, the structural basis of this reduced side-effect profile was interrogated with structural docking studies where alprazolam was shown to bind more strongly to the α1β3γ2L $GABA_A$ receptor compared to KRM-II-81 (Witkin et al., 2020).

Respiratory depression is another potential medical liability of GABAkines when given with respiratory depressing drugs like opioids. In multiple measures of respiratory function, KRM-II-81 was either inactive or less potent than alprazolam (Witkin et al., 2019a).

GABAkines can impair memory and cognitive function (Crowe and Stranks, 2018; Engin et al., 2018). Indeed, some benzodiazepines like midazolam are used as pre-anesthetic sedatives where they convey the useful side effect of surgical memory loss (Kim et al., 2015; Patel and Kurdi, 2015). In one study evaluating potential cognitive impact, midazolam reduced spontaneous alternation in a T-maze from 70 to 33% whereas KRM-II-81 did not significantly reduce this measure of cognition (61%) (Lewter, 2019).

b. Dependence and Abuse liability. 1,4-Benzodiazepine anxiolytics can be abused (Griffiths and Wolf, 1990; Woods et al., 1992) and are legally scheduled as controlled substances for this reason. The U.S. FDA updated its boxed warning for this class of compounds in September 2020 and warned about the safety issues concerning their combined use with opioids (Hirschtritt et al., 2021). Diazepam was compared to KRM-II-81 in a model that has predictive validity for abuse liability. Intracranial self-stimulation (ICSS) of the medial forebrain bundle is altered by drugs of abuse (Negus and Miller, 2014). In contrast to diazepam, KRM-II-81 did not facilitate ICSS behavior in normal or acid-induced pain-treated rats (Moerke et al., 2019).

Another study used drug discrimination to evaluate the potential abuse liability of KRM-II-81. Drug discrimination methods are another primary assay system with which judgments of abuse liability are made (Balster, 1991). Rats discriminated midazolam from control after 20 sessions but did not discriminate KRM-II-81 from control even after 100 sessions of training(Lewter, 2019). In a comparable study with TPA023B, another GABAkine that selectively avoids impacting α1-containing GABAARs, discriminative control of behavior in rats was not achieved even over a regimen of 160 experimental training sessions (Kohut and Ator, 2008). Moreover, TPA023B did not fully substitute in rats trained to discriminate zolpidem or lorazepam from vehicle (Kohut and Ator, 2008).

Drug self-administration is one of the primary methods to screen for potential abuse liability of drugs (Ator and Griffiths, 2003). In baboons,

the α2/3-selective GABAkine, TPA023, did not maintain self-administration in contrast to lorazepam and TPA123, a compound without selectivity over α1-containing GABAARs. Lack of self-administration occurred despite full occupancy of GABAARs as measured by displacement of (^{11}C)flumazenil binding (Ator et al., 2010).

When a high dose of TPA023 (32 mg/kg/24 h) was given to baboons by continuous intragastric drip, no physical dependence was observed. Only mild withdrawal signs or one moderate sign (retching/vomiting) was observed when the antagonist flumazenil was given at day 14 or when TPA023 dosing was abruptly stopped after 30 days (Ator et al., 2010). These findings contrast with the marked withdrawal signs observed with 1,4-benzodiazepines (Ator et al., 2000). No data currently exist to inform the relative ability of KRM-II-81 to induce physical dependence.

D. Early-Stage Compounds

Medicinal chemistry efforts to identify improved structures for GABAAR ligands is ongoing. This research enterprise has brought new compounds into use as tools to identify biological bases for disease and has led to increased understanding of the structural and molecular underpinnings of key drug-protein interactions. Importantly, these newer compounds stand as potential novel compounds from which the next breakthrough GABAkines will come. An excellent review of newly-patented compounds is available (Crocetti and Guerrini, 2020). A few highlights of early-stage compounds will also be reviewed here.

Reports have emerged on a new compound that is a selective GABAkine of α3-containing GABAARs. A study from Rowlett's lab (Meng et al., 2020) showed that YT-III-31 (8-ethynyl-*N*-methyl-6-phenyl-4*H*-benzo[*f*]imidazo[1,5-a][1,4]diazepine-3-carboxamide) did not increase punished responding of Rhesus monkeys, a finding that is opposite to that of the anxiolytic-like effects found with traditional benzodiazepine anxiolytics and with the α2/3-GABAkine, KRM-II-81 outlined earlier in this review. Instead, YT-III-31 had unique sedative-like effects. These findings are consistent with the idea that α2 but not α3-containing GABAARs (for which YT-III-31 is preferening) are pivotal for driving anxiolytic-like effects. Another study from Rowlett's lab showed that in Rhesus monkeys discriminating ethanol, only triazolam (non-selective), an α5-preferring GABAkine, and an α2/3/5-preferring GABAkine fully reproduced ethanol like discriminative stimulus effects. In monkeys self-administering ethanol orally, an α2/3-(HZ-166) and an α3-preferring (YT-III-31) GABAkine facilitated ethanol but not sucrose drinking (Berro et al., 2021). A study in rats from by the Savić lab showed that YT-III-31 was able to produce anxiolytic-like efficacy in the open field and elevated plus-maze assays at low, non-sedating doses (Batinić et al., 2018). They also did work to help identify the in vivo activity of this new compound. They used brain exposures to estimate in vivo potentiation of specific GABAAR configurations: YT-III-31 at doses

that were anxiolytic-like in rats did not potentiate α1βγ2 GABAARs. Their estimation also revealed only a modest selectivity of YT-III-31 for α3γ2 over α2γ2 and α5γ2 sites.

A new GABAkine for the $GABA_B$ receptor was recently disclosed and studied in vivo (Lobina et al., 2021). A novel compound with agonist and allosteric modulatory properties was reported (Olander et al., 2018). Etherington et al. (2017) reported some of the pharmacological activities of a novel α5-GABAkine acting at extrasynaptic GABAARs. A new chemical series of 1,2,3-triazolo-benzodiazepine derivatives were shown to be anticonvulsant against pentylenetetrazol and maximal electroshock (Shafie et al., 2020). A new series of 11-dialkylaminomethyl-2,3,4,5-tetrahydrodiazepino[1,2-a]benzimidazoles with potent anxiolytic properties was reported (Maltsev et al., 2021). New analogs of 4,6-diphenylpyrimidin-2-ol were described that identified a new potent compound active in vivo (Khoramjouy et al., 2021). 5-(2-aryloxy-4-nitrophenyl)-4H-1,2,4-triazoles and 5-(2-aryloxy-3-pyridyl)-4H-1,2,4-triazoles, possessing C-3 thio or alkylthio substituents were identified and found to be potent anticonvulsants (Navidpour et al., 2021). A new series of 3-{2-[1-acetyl-5-(substitutedphenyl)-4,5-dihydropyrazol-3-yl]hydrazinylidene}-1,3-dihydro-2H-indol-2-ones were reported with anticonvulsant activity (Kerzare et al., 2021). The design and synthesis of three different series of 1,5-benzodiazepines substituted at the 2 and 4 position were reported with a lead compound having diazepam-like high potency and drug-like properties (Verma et al., 2020). In another effort to identify novel structural GABAkines, Nilkanth et al. (2020) reported the synthesis and anticonvulsant activity of a new 1,3-dihydro-2H-1,4-benzodiazepin-2-one azomethines and 1,3-dihydro-2H-1,4-benzodiazepin-2-one benzamides; the lead compound was reported to be more efficacious than diazepam under their assay conditions and with lead-like properties. A new series of 4-phenyl-6H-imidazo[1,5-a]thieno[3,2-f][1,4]diazepine-7-carboxylate esters were synthesized by Di Capua et al. (2020) and shown to have in vivo activity in a range of benzodiazepine-detecting assays systems; some compounds were active without notable ancillary side-effects. Zolpidem and alpidem were used as standards to create new imidazopyridines. Tikhonova et al. (2020). The isosteric replacement of the pyridine nucleus by 1,3-thiazole, 1,3,4-thiadiazole, or 1,3-benzothiazole brought about new GABAkines.

Pandey et al (2020) reported new analogs of HZ-166. These compounds used 2,4-disubstituted oxazoles and oxazolines as bioisosteric replacement of the ester function with the goal of improving oral bioavailability. Two new structural analogs, LKG-I-70 and KPP-III-5, were shown to be devoid of motor-impairing effects when given up to 100.

MIDD0301 (Yocum et al., 2019) was reported by Forkuo et al. (2018) to be a GABAkine with selective amplification at $\alpha_{1\text{-}3,5}\beta_3\gamma_2$ GABAARs. The compound was orally active with a long half-life. Their data suggest that MIDD0301 represents a new candidate compound that relaxes airway smooth muscle, reduces lung inflammation and mitigates airway hyper-responsiveness in a mouse model of asthma.

Newer methods are also being introduced to help identify molecular substrates of compound binding to and functional activation of GABAARs as well as the identification of novel drug therapies. For example, in a recent study by Crocetti and colleagues (2021) 8-methoxypyrazolo[1,5-a]quinazolines were examined with proximity frequencies analyses (quantification of the frequencies that a compound intercepts two or more amino acids in the process of binding). Their work led to the elucidation of a combination of amino acids αVAL203- γTHR142 and αTYR 160- γTYR 58 that could predict GABAkine function. Sansolone et al. (2019) described new photochemical methods to interrogate individual GABAARs, a technology that should have important consequences for the design of improved GABAkines. Structural studies (Iorio et al., 2020) on a newer series of pyrazoloquinolinones gave some insights into the structural dynamics of the pharmacophore that might help guide new compound discovery. A new potential drug target for diazepam-refractory status epilepticus was announced - proinflammatory cytokine high mobility group box-1 (HMGB1) (Zhao et al., 2020). Along with this, Burman et al. (2019) reported on other potential mechanisms of diazepam resistance. New compounds have been used to help identify new binding domains for neuroactive steroids (Yu et al., 2019; Jayakar et al., 2020). Another auxiliary protein associated with GABAARs was also recently disclosed. Shisa7 regulates GABAAR trafficking, function, and pharmacology relevant to the control of GABAkine activity (Han et al., 2020).

A recent review of the Translocator Protein 18 kDa (TSPO), long-postulated to be a binding site for benzodiazepine anxiolytics is now available (Barresi et al., 2021). Alpidem was used as a prototype to create novel structures interacting with the TSPO binding site (Gudasheva et al., 2020). The dipeptide, GD-102 (*N*-phenylpropionyl-l-tryptophanyl-l-leucine amide), was shown to produce potent anxiolytic-like effects in vivo that were blocked by a TSPO binding site antagonist.

VI. Conclusions.

For well over a century, GABAkines (e.g., barbiturates) have been important and highly used medicines with diazepam being on the list of Essential Medicines of the World Health Organization. Efforts to discover and develop improved compounds for therapeutic use has been ongoing. This work has led to an array of pharmacological tools for research into the neurobiological substrates of disease and for disease therapeutics.

As noted throughout the current review, GABAkines can produce a host of undesirable side-effect and safety issues including sedation, memory-impairment, tolerance, dependence, and abuse. It remains to be seen whether the newer GABAkines reviewed here will, as hoped, display reduced liabilities.

Several new GABAkines are currently in development including neuroactive steroids, and an α2/3-preferring (KRM-II-81) and an α2/3/5-preferreing GABAkine (PF-06372865). The neuroactive steroids are in clinical development for depression and intractable epilepsy with clinical studies directed also at other indications. PF-06372865 (darigabat, formerly CRV-865) appears to be in development for epilepsy and anxiety.

KRM-II-81, a non-benzodiazepine GABAkine, is one of the newest GABAkine to enter development and is currently in the late preclinical phase. Preclinical pharmacological studies of KRM-II-81 have demonstrated its efficacy in animal models of anxiety, depression, acute and chronic pain, epilepsy, and traumatic brain injury. Preclinical data also support the potential for reduced tolerance and dependence. The efficacy of KRM-II-81 in models of pharmacoresistant epilepsy, preventing the development of seizure sensitization, and in brain tissue of intractable epileptic patients bodes well for improved therapeutics. The data on these newer GABAkines highlight the possibility of developing improved medicines in areas of clinical need.

Footnote

[1]This manuscript is dedicated to Dr. Michael A. Rogawski for his pioneering work on neuroactive steroids and other GABAkines and his continued devotion to bring improved medicines to patients in need.

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Conflict of interest

James Cook and Michael Poe are named as inventors on patents describing KRM-II-81 and analogs, certain rights to which have been licensed to RespireRx Pharmaceuticals Inc. Rok Cerne, Michael Poe, James Cook, and Jeffrey Witkin are members of the research advisory group for RespireRx Pharmaceuticals Inc and Arnold Lippa serves as Executive Chairman and Chief Scientific Officer.

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