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**ABSTRACT**

Enhancement of GABA<sub>A</sub> receptor inhibition has long been used in the treatment of anxiety beginning with meprobamate, diazepam, chlordiazepoxide, and alprazolam in present times. Positive allosteric modulation of GABA<sub>A</sub> receptors has thus proven its place in medical practice. Subsequent work focused on the design of compounds with reduced sedative liabilities. Several non-benzodiazepine GABA<sub>A</sub>-positive allosteric modulator (PAM) compounds (MRK-409, TPA-023, TPA-0238, NS11821, AZD7325 and PF-06372865) were tested in early clinical trials but suffered from signs of sedation and motor impairment and only three compounds progressed to proof of concept studies (TPA-023, AZD7325 and PF-06372865). TPA-023 was terminated due to toxicity in preclinical species while AZD7325 and PF-06372865 did not achieve efficacy endpoints in clinical trials. All compounds tested in Phase-II trials produced some signs of sedation at the minimum effective dose. We highlight a new compound, KRM-II-81, that is an imidazodiazepine selective for GABA<sub>A</sub> receptors containing α 2/3 proteins. KRM-II-81 has demonstrated a reduced liability for motor-impairing and respiratory effects compared to non-selective agents. KRM-II-81 has shown efficacy in animal models of epilepsy and is active in models for which other standard-of-care antiepileptics are not active. KRM-II-81 also produces anxiolytic-like effects but with minimal sedation. In contrast to benzodiazepines like diazepam, KRM-II-81 also produces anti-nociceptive effects including reduction in pain responses in models of neuropathic pain. Unlike diazepam, KRM-II-81 displays antidepressant-like effects. KRM-II-81 dampens cortical excitability in mice with traumatic brain injury. Thus, KRM-II-81 is a newly discovered, non-benzodiazepine compound, which targets a selective population of GABA<sub>A</sub> receptors for improved therapeutic gain and reduced side effects.

**ABBREVIATIONS**

KRM-II-81: 5-(8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-3-yl)oxazole; PAM: Positive Allosteric Modulator
INTRODUCTION

The history of diazepam rests upon the backs of giants. The first rationally-designed anxiolytic drug, meprobamate (Miltown) (Figure 1) was discovered and championed by Frank Berger who modified (with Bernard Ludwig) the muscle relaxant mephenesin with the goal of reducing muscle-relaxing and sedative properties while augmenting anti-anxiety effects. Miltown was the first blockbuster drug and was, in the late 1950s, being used by many people in the United States [1]. 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 [2]. The lead of medicinal chemistry, Leo Sternbach directed animal testing [3] and took the compound himself providing the first clinical data on chlordiazepoxide [2]. With the introduction of chlordiazepoxide (Librium) (Figure 1) into clinical practice with FDA approval in 1959, another generation of anxiolytic agents was born and, as with meprobamate, found widespread use for anxiety. Diazepam (Valium) (Figure 1) 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 [4]. Valium was and still is a highly valuable drug used for the treatment of anxiety and other disorders including acute convulsions. Despite its bad press for being addictive [5], and the reluctance of the medical community to prescribe it wholesale, it is still widely used and is sold over-the-counter in a number of countries and has been included in the World Health Organization’s List of Essential Medicines. Valium as an anxiolytic has now been largely supplanted by another benzodiazepine, alprazolam [6,7] (Figure 1).

Overall, this history demonstrates the huge demand for medicines that control anxiety, a disorder of high prevalence worldwide. In modern times, primarily due to concern for dependence and abuse of benzodiazepine anxiolytics, the first-line therapies for anxiety prescribed by most physicians in the United States are the antidepressant/anxiolytics that block reuptake of monoamines (e.g., selective serotonin uptake inhibitors or SSRIs like Prozac, Figure 1). Although there is ample clinical documentation of their ability to impact anxiety symptoms [8,9], the comparative magnitude of effect is often relatively small, it requires weeks of daily dosing to achieve full therapeutic benefit in responders [10-12] and can lead to adverse effects such as sexual dysfunction in some patients [13].

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 the dose-
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α2/3-Containing GABAA Receptors: Preclinical Data Predict Enhanced Efficacy for Epilepsy, Chronic Pain, Anxiety, and 

dependent sedation that these compounds produce. While sedation is sometimes desired, sedation is a dose-limiting side-
effect for some other indications. 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. This major point will be 
elaborated below in the discussion of the comparative 
pharmacology of diazepam vs. a newly discovered GABAA 
receptor PAM.

Rational drug discovery efforts directed at creating improved 
GABAA receptor PAMs came from basic pharmacological data 
along with the discovery of the benzodiazepine receptor 
[14,15] and its role in potentiation of GABA currents [16]. This 
discovery enabled establishment of binding assays (using 
[3H]BZs to identify and optimize ligands for benzodiazepine 
receptor interaction [17]. Promising ligands were then 
evaluated in animal models for efficacy and reduced 
unwanted side effects (reviewed in [18]). At least four such 
compounds (bretazenil, abecarnil, alpidem, and ocinaplon) 
progressed into clinical trials due to their favorable preclinical 
profile but mostly discontinued due to sedation (bretazenil, 
abecarnil) or liver toxicity (ocinaplon) observed in humans. 
Alpidem was approved as an anxiolytic with relatively little 
separation [19] but was later withdrawn due to high occurrence 
of hepatitis [20]. Based upon the ability of some compounds to 
produce anxiolytic-like effects without sedation in animal 
models (e.g., CL218-872), it was early hypothesized that 
multiple benzodiazepine receptors might exist that mediate 
anxiolytic versus sedative effects [21]. The advent of molecular 
biology enabled further refinement in the search for anxiol-
selective drugs.

The GABAA receptor is a pentameric ligand-gated ion channel, 
allowing for various combinations of five different subunits, 
which are expressed humans as the following types: α 1-6, β1-
3, γ1-3, δ1-3, ε, π, and θ [22,23]. Each functional GABAA 
receptor includes both an α and β subunit, and typically 
include α-, β-, and γ-subunits in a 2:2:1 ratio for functional 
activity. The particular α-subunit contributing to the 
benzodiazepine binding site of GABAA receptors defines the 
receptor’s pharmacological properties; α1-subtype-containing 
GABAA receptors have been found to preferentially mediate 
the sedative, amnestic, ataxic effects of ligands as well as 
dependence [24-29], whereas α2- and α3-subtypes mediate 
anxiolytic effects [30,31] and pain therapeutics [32,33] and 
the α5-subtype has been implicated in memory function 
[34,35]. Such studies also directly demonstrated that when the 
α1-subtype was rendered insensitive to benzodiazepines, the 
therapeutic window of diazepam was markedly increased [30] 
while the anxiolytic efficacy of diazepam was retained [25]. In 
addition, analgesic efficacy, not previously observed with 
diazepam, was uncovered due to the decreased sedation and 
motor impairment that resulted from the deletion of its 
interactions with α1-containing GABAA receptors [36]. Based 
primarily on the data associating α1-containing GABAA 
receptors with sedation, substantial discovery effort over the 
last 15 years was directed at the identification and 
development of GABAA-receptor PAM anxiolytics, antiepileptics and anxiolytics with preference for α2 and α3 
over α1-containing GABAA receptor subtypes [37].

**SUBTYPE SELECTIVE GABAA PAMS**

One of the first “selective” molecules reported was L-838,417 
(Figure 2), a partial agonist at α2,3- and α5-containing 
receptors 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 [25,38]. 
Further drug discovery effort at Merck resulted in three 
compounds which were progressed into clinical studies; two 
analogs of L-838,417 (TPA-023 and MRK-409) (Figure 2) 
and a structurally unrelated TPA-023B (Figure 2) [39]. All three 
compounds were partial agonists at α2/3 subtypes with no 
substantial α1 efficacy in vitro [40]; they were all efficacious in 
animal models of anxiety without observed sedation [41]. 
Clinical data, however, presented a more complex picture - 
MRK-409, despite its minimal activity at α1 subtypes, 
produced sedation in man at relatively low (< 10%) levels of 
receptor occupancy [42]. Considering that the sedation liability 
of MRK-409 in man could be attributed to its residual partial 
anxiolytic efficacy at the α1 subtype [43], a second compound in
this series, TPA-023, was developed which lacked any appreciable efficacy at the α1 subtype. In Phase-II clinical trial, TPA-023 produced anxiolytic effects, however it also exhibited signs of sedation such as dizziness, drowsiness, and motor incoordination [41,44-46]. The sedative effects were however observed at relatively high levels of receptor occupancy (>50%) which was substantially higher than 24% reported for diazepam [47]. The clinical trial was terminated early due to preclinical toxicity issues (cataract formation in rodents) which prevented completion of the study and determination of a conclusive efficacy readout. It is possible that mild sedative effects of TPA-023 are at least in part due to the potentiation of GABAA α 2/3 subtypes as reported in a recent primate study [48]. The follower compound, TPA-023B, which similarly lacked α1 PAM activity in vitro (was an antagonist) produced weak signs of sedation in early clinical trials at approximately 50% receptor occupancy [49]. No human efficacy data were reported and clinical development of TPA-023B was terminated. The reasons for this decision were not publicly disclosed.

Two more recent α1-sparing, partial subtype-selective GABAA receptor PAMs are NS11821 from Neurosearch (structure not disclosed) which primarily potentiates α2/3/5 subtypes and AZD7325 from Astra Zeneca (Figure 2) which exhibited good efficacy at α2/3 subtypes. In animal models both ligands produced a dose dependent reduction of anxiety-like behavior with less sedation, motor impairment, and memory impairment than diazepam or chlor diazepoxide [50,51]. In early clinical trials NS11821 displayed a small pharmacodynamic effect (a decrease in saccadic peak velocity) with weak signs of sedation (body sway and the visual analogue scale for alertness) and signs of memory impairment which may result from its activity at α5 subtypes [51]. No receptor occupancy (RO) was reported for NS11821 and the compound was not evaluated in a proof of concept clinical trial. AZD7325 required RO > 80% [50,52] to produce a pharmacodynamic response (saccadic peak velocity, EEG spectrum); however, this high level of receptor occupancy was not sufficient for achieving significant anxiolytic activity [50]. While AZD7325 produced lower cognitive and neurophysiological side effects than lorazepam, benzodiazepine-like side effects (dizziness, headache and somnolence) were reported at sub-anxiolytic doses [50].

The most recent GABAA receptor subtype selective compound (α2/3/5 vs α1) evaluated in the clinic was PF-06372865 (Figure 2) [53,54]. PF-06372865 was efficacious in an animal model of absence epilepsy [55] in multiple pain modalities in a Phase-I clinical trial [56], and in a small Phase-II trial for photosensitive epilepsy [57]. No severe side effects were reported, although sedation and dizziness were reported in half of the photosensitive epilepsy patients. When tested in a larger Phase-II trial for lower back pain, the ligand did not achieve the primary efficacy end point of reduction in pain intensity and produced benzodiazepine-like side effects including sedation and memory impairment [58]. In a clinical Phase-II trial for anxiety, PF-06372865 failed for lack of efficacy and for induction of side effects [59]. It is possible that the ~50% occupancy produced with the maximal dose of 7.5 mg was not sufficient for critical therapeutic effect [53]; however, the occurrence of somnolence, dizziness and memory impairment at this dose would preclude higher dosing. No further work on this compound has been reported.

In summary, all of the compounds with relative in vitro preference for α2/3 versus α1-containing GABAA receptors displayed efficacy in the absence of benzodiazepine-like side effect in animal models. However, both the efficacy and the side-effect profiles in humans were not as impressive as preclinical data forecasted. Benzodiazepine-like side effects were observed for all compounds in early clinical trials and only three compounds progressed to Phase-II (TPA-023, AZD7325 and PF-06372865) where they suffered from weak efficacy at the dose that started to produce side effects. These findings suggest that reduced activity at α1-containing GABAA receptors, while beneficial, has not been sufficient to create the desired therapeutic profile for the drug developers to date.

**BIASED BENZODIAZEPINE RECEPTOR LIGANDS**

Even though benzodiazepines as a class act at all γ subunit containing GABAA receptors (α1,2,3,5), some compounds display less sedation than others. One such compound is clobazam [60,61], whose milder sedative liability could have contributed to its approval as an add-on therapy for Lennox-Gastaut syndrome [62]. A small proof of concept clinical trial
also reported reduction of capsaicin-induced hyperalgesia with clobazam [63]. The activity of clobazam might be due, at least in part, to buildup of its active metabolite, N-desmethyl-clobazam. The metabolite exhibits functional selectivity for α2,3,5 GABAα receptor subtypes and is less efficacious at α1 subtype [64]. N-desmethyl-clobazam was further evaluated in vivo in animal models of pain where it produced significant analgesia without sedation [64] The authors of the study filed a patent for clinical use of N-desmethyl-clobazam for chronic pain [65] but the compound has not, to our knowledge, been evaluated in a clinical setting. The discovery of compounds which retain the beneficial properties of benzodiazepines but cause less sedation continues to be an exciting proposition.

Cook and associates synthesized HZ-166 (Figure 3), a non-benzodiazepine molecule [imidizodiazepine] GABAα receptor PAM with preference for α2 and α3 versus α1-containing GABAα receptor subtypes [66,67]. The selectivity of HZ-166 compared to diazepam is shown in (Figure 4). HZ-166 was efficacious in animal models of pain and produced no overt sedation or tolerance [68]. Further progression of HZ-166 was prevented by its poor pharmacokinetic properties resulting from the ester functionality rendering the compound liable to metabolic deactivation through ester hydrolysis [e.g., Poe et al. [67]]. This liability led to SAR optimization and the synthesis of several HZ-166 analogs with improved pharmacokinetic properties. One such analog, KRM-II-81 (Figure 3) was discovered in 2016 when Poe and colleagues created the oxazole bio-isostere of HZ-166, KRM-II-81, by a straightforward synthetic route (Figure 5). KRM-II-81 retained selectivity for GABAα α2/3 receptors over GABAα α1 receptors expressed in oocytes [33,69] (Figure 4). In contrast, diazepam does not largely discriminate among GABAα receptor configurations (Figure 4). Similar observations were reported for GABAα receptors expressed in mammalian cells where both the potency and efficacy of KRM-II-81 at the α1 subtype were lower than at α2 and 3 subtypes [67]. Conversely, the reverse was reported for zolpidem with higher efficacy and potency at the α1 subtype [70].

The physiological relevance of the effects of KRM-II-81 was further demonstrated in isolated dorsal root ganglion neurons where KRM-II-81 potentiated native GABA currents [71].

Diazepam is a known anxiolytic [79]. The GABAA receptor α2/3 mechanism is also effective in producing anxiolytic-like effects. For example, KRM-II-81, like the anxiolytic chlordiazepoxide, decreased marble-burying in mice (Figure 8, left panel). However, in contrast to KRM-II-81, chlordiazepoxide impaired motor performance of these mice on a rotarod (Figure 8, right panel).

Benzodiazepine anxiolytic drugs are not generally used for the treatment of Major Depressive Disorder (MDD). KRM-II-81 was active in the forced-swim test in mice, a model that detects antidepressant drugs (Figure 9). In contrast, diazepam was not active under these conditions. However, if the motor-impairing effects of diazepam are prevented by the α1-containing models of inflammatory pain [33] and in models of neuropathic pain [71].

Figure 6. Left Panel. Comparative effects of HZ-166, KRM-II-81, KRM-II-82, and diazepam against convulsions induced by pentylenetetrazole (PTZ, i.v.) in rats. Data show the dose of PTZ required to induce convulsions as a function of drug dose (mean ± SEM, n=8). * p<0.05 compared to vehicle by Dunnett’s test after ANOVA. Right Panel. Comparative effects of HZ-166, KRM-II-81, and diazepam on after-discharge thresholds in amygdala-kindled rats. The scale for the after-discharge threshold (ADT) is scale-adjusted to capture the stimulation scale change required to observe a seizure from the previous baseline to the ADT scored on test day. The average scale adjusted ADT was approximately 0.63 mA in vehicle treated rats. * p<0.05 compared to vehicle control (n=8/group) by Dunnett’s test after ANOVA Reprinted from Neuropharmacology, Vol. 137, Witkin et al. [72], Bioisosteres of ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[fimidazo [1,5-a][1,4]diazepine-3-carboxylate (HZ-166) as novel α2,3 selective potentiators of GABA_A receptors: Improved bioavailability enhances anticonvulsant efficacy. Pages 332–343, Copyright © 2018, with permission from Elsevier Ltd.
GABAA receptor antagonist, B-CCt [74], then diazepam showed an antidepressant-like signal comparable to that of KRM-II-81 (Figure 9).

That α1-containing GABAA receptors are responsible for the motor-impacting effects of GABAA receptor PAMs was further supported by data comparing KRM-II-81 with KRM-II-82. KRM-II-82 impaired rotarod performance of mice at 30 mg/kg whereas KRM-II-81 did not (Figure 10). KRM-II-82 potentiated current in GABAA receptors containing α1 subunits, whereas KRM-II-81 did not (Figure 4).

Side effects of drugs are only important when considered in relationship to their therapeutic or efficacious doses or exposure levels. Diazepam and KRM-II-81 can be contrasted based upon a ‘therapeutic index’. For example, when doses that impair motor performance are compared to the doses that produce efficacy in rodent seizure models, KRM-II-81 showed a larger separation or protective index than that of diazepam across a host of assays (Figure 11). A similar separation in respiratory side-effects has been reported between KRM-II-81 and the anxiolytic alprazolam (Figure 12).

Figure 7: Comparison of effects of diazepam and KRM-II-81 on pain behaviors in the late phase of the formalin assay in rats. Effects of tramadol (Tram, 80 mg/kg) are shown as a positive control. Each point represents the mean ± SEM of the same 8 rats *p<0.05 compared to vehicle control data by Dunnett’s test after ANOVA. Data are extracted and replotted from Witkin et al. [71].

Figure 8. Left Panel. Effects of KRM-II-81 and chlordiazepoxide (CDAP) on marble-burying in mice. Data represent the mean ± SEM (n=10). * p<0.05 compared to vehicle control by Dunnett’s test after ANOVA. Right Panel. Effects of KRM-II-81 and chlordiazepoxide on rotarod performance of mice. Data represent the mean ± SEM (n=10). KRM-II-81 and chlordiazepoxide were given at 30 mg/kg. * p<0.05 compared to vehicle control by Dunnett’s test after ANOVA. Data are extracted and replotted from Li et al. and Poe et al. [67].

Figure 9: Effects of KRM-II-81 (10 and 30 mg/kg, i.p.) and diazepam (22 mg/kg, p.o.) alone or in the presence of B-CCt (10 mg/kg) on immobility time in the forced-swim test in mice. Data represent the mean ± SEM (n=7-8), * p<0.05 compared to vehicle control by Dunnett’s test after ANOVA. Data are extracted and replotted from Methuku et al. [69].
The idea that α protein composition of GABAA receptors can guide pharmacological effects has been key to the rational discovery of the compounds discussed above. However, it should be recognized that there are aspects of the biology and pharmacology of GABAA receptors that are not fully understood. The mechanism of sedation appears to be more complex than the singular reliance on α1 proteins. For example, a recent study in primates demonstrated that anxiolytic compounds with functional selectivity for α2, α3, and/or α5 GABAA receptors can produce a mild-type of sedation through the activation of the same receptors subtypes [48]. In addition there is evidence that some of the sedation and ataxia mediated by drugs that fell out of the clinic was mediated by positive modulation at α5/β3/γ2 subtypes [80-82]. The non-sedating profile of KRM-II-81 is therefore consistent with the lack of α5 GABAA receptor potentiation observed invitro [33,69]. A different study using α1-subtype diazepam insensitive mice [α1 (H101R) mice] found no difference in the effect of diazepam on sleep EEG between the mutant and the wild type mice [83]. Considering that sleep can be used as sedation biomarker [18] the data suggest that the α1 subtype of GABAA receptor is not the exclusive driver of sedation.

We suggest that there are likely multiple routes and mechanisms by which one can design molecules to achieve medically-targeted effects while reducing side-effects. KRM-II-81 appears to achieve this end through its selectivity to α2/3-containing GABAA receptors. Ocinaplon, in contrast, does not selectively target these α proteins but has been shown in the clinic to have a reduced sedative liability [84,85]. Other methods may also exist for optimized drug efficacy/side-effect balances within this system. For example, β2/3-subunit

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Figure 10: Effects of KRM-II-81 and KRM-II-82 on rotarod performance of mice. Data represent the mean (n=10). * p<0.05 compared to vehicle control. Data are extracted and replotted from Poe et al. [67].

Figure 11: Protective indices for different anticonvulsant measures in rats. The protective index is the ratio [minimal effective dose impairing motor performance/minimal effective anticonvulsant dose]. KRM: KRM-II-81; Dzp: diazepam. Data are extracted and replotted from Witkin et al., [72].

Figure 12: Effects of KRM-II-81 compared to alprazolam on respiration in rats. Both compounds were dosed at 3.2 mg/kg. Data are means + SEM (n=8). Respiration rate was measured as breaths/min and respiratory volume was tidal volume/Kg. * p<0.05 compared to vehicle control by Dunnett’s test after ANOVA. Data are extracted and replotted from Witkin et al. [71].
subtype-selective GABAA receptor PAMs have been shown to produce reduced motor-impacting effects [86-88]. Finally, while lower sedative effects are desirable, their elimination is not necessarily required for an improved therapeutic agent. The benzodiazepine, alprazolam, is highly prescribed for anxiety despite sedative properties. Other areas of neurological and psychiatric practice are in such need of improved medications, that improved efficacy, as predicted for KRM-II-81, are likely sufficient for driving their development. For example, despite its dose-dependent induction of motor-impairment that includes falling, perampanel (Fycompa) is used as a newer antiepileptic drug [89]. And for pain, the opioids that have produced devastating health and morbidity consequences [90] are in clear need of replacement where efficacy with improved safety, as predicted for KRM-II-81, would be key developmental drivers.

In summary, KRM-II-81 produces biological effects in rodent models suggesting its therapeutic value in several neurological and psychiatric disorders. At the same time, KRM-II-81 can be differentiated from that of GABAA receptor modulators that are not selective for α2/3-containing GABAA receptor PAMs. The unique and improved efficacy of KRM-II-81 in models of epilepsy and in neuropathic pain is promising. The compound awaits clinical data to begin to evaluate the therapeutic potential of this novel GABAA receptor PAM.

Acknowledgments

We are grateful for the Henry and Nellie Pence family for supporting this work by a Pence foundation grant awarded to Jodi L. Smith. We are also grateful to John and Nancy Peterson for their support of this research.

We thank the National Institutes of Health for support from [MH-096463] and [NS-076517] and The National Science Foundation, Division of Chemistry [CHE-1625735]. We also acknowledge UW-Milwaukee’s Shimadzu Laboratory for Advanced and Applied Analytical Chemistry and support from the Milwaukee Institute of Drug Discovery and the University of Wisconsin-Milwaukee Research Foundation.

Conflict of interest

Authors report no conflict of interest with the exception that James M. Cook is a patent holder for the invention of KRM-II-81. The University of Wisconsin-Milwaukee owns the patent.

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