Chemical, Pharmacological, and Structural Characterization of Novel Acrylamide-Derived Modulators of the GABAA Receptor

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**Abbreviations:** DM480, (E)-3-(furan-2-yl)-N-(indolin-1-yl)acrylamide; DM489, (E)-3-(furan-2-yl)-1-(indolin-1-yl)prop-2-en-1-one; DM490, (E)-3-(furan-2-yl)-N-methyl-N-(p-tolyl)acrylamide; DM492, (E)-1-(3,4-dihydroquinolin-1(2H)-yl)-3-(furan-2-yl)prop-2-en-1-one; DM495, (E)-1-(3,4-dihydroisoquinolin-2(1H)-yl)-3-(furan-2-yl)prop-2-en-1-one; DM497, (E)-3-(thiophen-2-yl)-N-(p-tolyl)acrylamide; EC50, ligand concentration that produces half-maximal activation or potentiation; Emax, maximal efficacy; etomidate, R(+)-etomidate; GABAAR, γ-aminobutyric acid type A receptor; Gzn-OG, gabazine-oregon green; IC50, ligand concentration that produces half-maximal inhibition; K0, inhibitory constant; nAChR, nicotinic acetylcholine receptor; nH, Hill coefficient; PAM, positive allosteric modulator; PA, probability of being in the active state; PAM-2, (E)-3-furan-2-yl-N-p-tolylacrylamide; PAM-4, (E)-3-furan-2-yl-N-phenylacrylamide; PS, pregnenolone sulfate
Abstract

Acrylamide-derived compounds have been previously shown to act as modulators of members of the Cys-loop transmitter-gated ion channel family, including the mammalian GABA<sub>A</sub> receptor. Here, we have synthesized and functionally characterized the GABAergic effects of a series of novel compounds (termed "DM compounds") derived from the previously-characterized GABA<sub>A</sub> and the nicotinic α7 receptor modulator (E)-3-furan-2-yl-N-p-tolyl-acrylamide (PAM-2). Fluorescence imaging studies indicated that the DM compounds increase apparent affinity to the transmitter by up to 80-fold in the ternary αβγ GABA<sub>A</sub> receptor. Using electrophysiology, we show that the DM compounds, and the structurally-related (E)-3-furan-2-yl-N-phenylacrylamide (PAM-4), have concurrent potentiating and inhibitory effects that can be isolated and observed under appropriate recording conditions. The potentiating efficacies of the DM compounds are similar to those of neurosteroids and benzodiazepines (ΔG ~ -1.5 kcal/mol). Molecular docking, functionally confirmed by site-directed mutagenesis experiments, indicate that receptor potentiation is mediated by interactions with the classic anesthetic binding sites located in the transmembrane domain of the intersubunit interfaces. Inhibition by the DM compounds and PAM-4 was abolished in the receptor containing the α1(V256S) mutation, suggestive of similarities in the mechanism of action with that of inhibitory neurosteroids. Functional competition and mutagenesis experiments, however, indicate that the sites mediating inhibition by the DM compounds and PAM-4 differ from those mediating the action of the inhibitory steroid pregnenolone sulfate.
Significance Statement

We have synthesized and characterized the actions of novel acrylamide-derived compounds on the mammalian GABA_A receptor. We show that the compounds have concurrent potentiating effects mediated by the classic anesthetic binding sites, and inhibitory actions that bear mechanistic resemblance to, but do not share binding sites with, the inhibitory steroid pregnenolone sulfate.
Introduction

γ-Aminobutyric acid type A receptors (GABA\(_A\)Rs) are members of the pentameric Cys-loop transmitter-gated ion channel family and, together with glycine receptors, are the principal inhibitory receptor-channels in the nervous system. The major physiological function of these receptors is maintaining the balance between inhibitory and excitatory neurotransmission. Dysfunction and dysregulation of GABA\(_A\)Rs can upset the equilibrium between inhibition and excitation in the brain, which may progress into pathological conditions, including depression, anxiety, insomnia, autism, epilepsy, memory impairment, schizophrenia, and addiction (Deidda et al., 2014; Hines et al., 2012; Tang et al., 2021). Accordingly, drugs that modulate GABA\(_A\)R function can be important clinically.

There is a long history of the medical use of GABA\(_A\)R potentiators in the treatment of some of these illnesses, including anxiety, epilepsy, insomnia as well as for sedation and anesthesia in clinical procedures (Chua and Chebib, 2017; Saari et al., 2011; Sieghart and Savic, 2018; Sigel and Ernst, 2018; Weir et al., 2017). However, not only the clinical efficacies of some of these medications are borderline but they also can be abused leading to dependence and addiction, making evident the need for new, safer medications.

To develop novel GABAergic agents, a series of acrylamide-based compounds (termed "DM compounds") were synthesized (Fig. 1). The DM compounds are structural analogs of (E)-3-furan-2-yl-N-p-tolyl-acrylamide (PAM-2), which potentiates the α7 nicotinic receptor (Arias et al., 2020) and several subtypes of the GABA\(_A\)R (Arias et al., 2022). Here, we have investigated the GABAergic pharmacological activities of the novel compounds, using fluorescence imaging and two-electrode voltage-clamp electrophysiology. To determine the location and structural components of the binding sites involved in positive and negative modulatory activity of DM compounds, site-directed mutagenesis, molecular docking and molecular dynamics (MDs) studies were performed on the α1β2γ2L GABA\(_A\)R. The rat α1β2γ2 GABA\(_A\)R model was constructed using the 3D structure of the human α1β2γ2 GABA\(_A\)R (Kim et al., 2020). The study is exploratory by nature. The findings are reported according to the guidelines detailed in (Michel et al., 2020).
The main findings of the study are that the tested DM compounds and the structurally-related (E)-3-furan-2-yl-N-phenylacrylamide (PAM-4) both potentiate and inhibit the GABA\(_A\)R at high micromolar concentrations. Receptor potentiation is mediated by the compounds acting at the classic anesthetic binding sites in the intersubunit interfaces whereas mutations to the second transmembrane domain strongly reduce inhibition by PAM-4.
Materials and Methods

Drugs

R(+)-Etomidate (etomidate) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). The salts used in buffers, HEPES, GABA, propofol, DMEM high glucose, fetal bovine serum, and 0.01% poly-L-Lys solution (70-150 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregnenolone sulfate was purchased from Bio-Techne Corp. (Minneapolis, MN, USA) and Sigma-Aldrich. Phenobarbital was obtained from J.T. Baker Chemical Co (Phillipsburg, NJ, USA). Lipofectamine 2000, Opti-MEM, and trypsin-EDTA were obtained from Thermo Fischer Scientific (Waltham, MA, USA). (E)-3-(furan-2-yl)acrylic acid, (E)-3-(2-thienyl)acrylic acid, 1-hydroxybenzotriazole hydrate (HOBt), p-toluidine, tetrahydroquinoline, tetrahydroisoquinoline and 1-aminoindoline hydrochloride were obtained from Sigma-Aldrich SRL (Milan, Italy). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), triethylamine, sodium hydride and iodomethane were purchased from Fluorochem (Glossop, UK). Gabazine-oregon green (Gzn-OG) was synthesized as described previously (Sakamoto et al., 2019).

Chemical synthesis of DM compounds

The method previously described for the synthesis of PAM-2 and DM489 (Arias et al., 2020) was applied for the synthesis of novel compounds and PAM-4 (Fig. 1). The procedure is given for the preparation of DM497. (E)-3-(2-thienyl)acrylic acid (1.0 mmol) was dissolved in anhydrous dichloromethane (5 mL) and the solution cooled to 0 °C. Then, triethylamine (2.0 mmol) and p-toluidine (1.0 mmol) were added, and the mixture was stirred for 1 h at 0 °C. Next, 1-hydroxybenzotriazole hydrate (HOBt) (2.0 mmol) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (2.0 mmol) were added, and the mixture stirred for 18 h at room temperature (RT). The mixture was then treated with dichloromethane (15 mL), and the organic layer washed with 6 N HCl (2 x 5 mL). After drying over sodium sulphate, the solvent was removed under reduced pressure, and the residue purified by silica gel flash chromatography.
[dichloromethane:methanol; 99:1 (v/v)], obtaining DM497 in 84% yield.

The same method was applied to obtain DM492 [(E)-1-(3,4-dihydroquinolin-1(2H)-yl)-3-(furan-2-yl)prop-2-en-1-one], DM495 [(E)-1-(3,4-dihydroisoquinolin-2(1H)-yl)-3-(furan-2-yl)prop-2-en-1-one], and DM480 [(E)-3-(furan-2-yl)-N-(indolin-1-yl)acrylamide], by coupling (E)-3-(2-furanyl)acrylic acid with tetrahydroquinoline, tetrahydroisoquinoline, or 1-aminoindoline hydrochloride, respectively. Silica gel flash chromatography was performed using hexane:ethyl acetate (8:2, v/v) for the purification of DM492 and DM495, which were obtained in 16% and 18% yield, respectively. The eluent for the purification of DM480 was dichloromethane:methanol:NH₄OH (99:1:0.1, by vol), and the compound was obtained in 67% yield.

For the synthesis of DM490 [(E)-3-(furan-2-yl)-N-methyl-N-(p-tolyl)acrylamide], PAM-2 was prepared as reported in Arias et al. (2020) and used as starting material. Briefly, 1.0 mmol PAM-2 was dissolved in anhydrous tetrahydrofuran (5 mL) and the solution cooled to 0 °C. Sodium hydride (2.0 mmol) was added, and the mixture stirred for 20 min at 0 °C. Then, iodomethane (3.0 mmol) was added and the mixture stirred for 3 h at room temperature. The mixture was next treated with dichloromethane (20 mL), the organic layer washed with water (2 x 5 mL). After drying over sodium sulphate, the solvent was removed under reduced pressure, and the residue purified by flash silica gel chromatography, with 50% yield.

**Molecular descriptors for DM compounds**

The molecular descriptors molecular volume, numbers of hydrogen bond donors and acceptors, lipophilicity (LogP) and estimates of brain penetration (LogBB) of the DM compounds were calculated using the Biovia Discovery Studio software (Dassault Systèmes Co., MA, USA). The results are provided for reference purposes in Table 1.

**Fluorescence imaging assays using GABA₄Rs transiently expressed in HEK293T cells**

Fluorescence imaging assays were conducted on the α1β3γ2L GABA₄R using gabazine-oregon...
green (Gzn-OG). Gzn-OG is a GABAAR antagonist that becomes fluorescent upon binding to the transmitter binding site (Sakamoto et al., 2019). Mouse α1, β3, and γ2L subunits were transiently transfected into HEK293T (CRL-3216, ATCC, Gaithersburg, MD, USA) cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) as described in detail previously (Sakamoto et al., 2019). Contamination for mycoplasma was not routinely tested. HEK293T-α1β3γ2L cells were incubated with 100 nM Gzn-OG for 5 min at 25 °C, followed by removal of the medium and wash with HEPES-buffered saline. The cells were then titrated with 0.01 µM to 30 mM GABA (three concentration points per decade) in the absence or presence of 10-100 µM of a DM compound.

Cell fluorescence imaging was performed using confocal laser scanning microscopy (CLSM) with a Carl Zeiss LSM-800 microscope (Carl Zeiss AG, Oberkochen, Germany), equipped with 63×, NA = 1.40 oil objective, and GaAsP detector. CLSM images were acquired using an excitation wavelength of 488 nm (0.75 %, Gain 750 V) and setting emission at 520 nm using the Definite Focus module included in LSM-800. Cell fluorescence was determined by enclosing the regions of interest. Non-specific fluorescence was determined at 30 mM GABA that was assumed to fully displace Gzn-OG. The values of fluorescence are given as mean ± S.E.M., from the specified number of cells (in all cases, ≥12).

Apparent IC50s for GABA were estimated from non-linear regression fitting of the pooled F/Fo ratios (KaleidaGraph 4.5, Synergy Software, Reading, PA, USA) using the following logistic equation:

\[
\frac{F}{F_o} = \frac{1}{1 + \left( \frac{IC_{50}}{[GABA]} \right)^{n_H}}
\]

(1)

where Fo and F are fluorescence intensity in the absence and presence, respectively, of GABA, [GABA] is the concentration of GABA, IC50 is the concentration of GABA that produces half-maximal inhibition of fluorescence, and n_H describes the slope of the curve. The fitted IC50s (best-fit
parameters ± standard error of the fit) were transformed to apparent $K_{d,GABA}$ (equilibrium dissociation constant) using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_{d,GABA} = \frac{IC_{50}}{1 + \frac{[Gzn-OG]}{K_{d,Gzn-OG}}}$$  \hspace{1cm} (2)

where $[Gzn-OG]$ is the initial concentration of Gzn-OG (100 nM) and $K_{d,Gzn-OG}$ (55 nM) is the $K_d$ for Gzn-OG (Sakamoto et al., 2019). The reported uncertainties for $K_{d,GABA}$ reflect error of the fit propagated through Eq. (2).

**Pharmacological activity of novel DM compounds at $\alpha1\beta2\gamma2L$ and $\alpha1\beta3\gamma2L$ GABA$_A$R subtypes**

Rat $\alpha1\beta2\gamma2L$ or human $\alpha1\beta3\gamma2L$ GABA$_A$Rs were expressed in *Xenopus laevis* oocytes as previously described (Pierce et al., 2022; Shin et al., 2017). The oocytes were purchased as quarter ovaries from Xenopus 1 (Dexter, MI, USA). The cDNAs for the $\alpha1$, $\beta2$, $\beta3$, and $\gamma2L$ in the pcDNA3 vector were linearized with XbaI (NEB Labs, Ipswich, MA, USA). The cRNAs were synthesized from linearized cDNA using mMessage mMACHINE (Ambion, Austin, TX, USA). The oocytes were injected with a total of 3.5 ng of cRNA per oocyte in the cRNA ratio of 1:1:5 ($\alpha$:$\beta$:$\gamma$). The $\gamma2L$ subunit cRNA was used in excess to enhance incorporation of the $\gamma2L$ subunit and to minimize the expression of binary $\alpha1\beta2$ or $\alpha1\beta3$ receptors. Following injection, the oocytes were incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES; pH 7.4), supplemented with 2.5 mM sodium pyruvate, 50 µg/mL gentamycin, 100 U/mL penicillin and, 100 µg/mL streptomycin, at 15 °C for 1-2 days prior to conducting electrophysiological recordings.

The electrophysiological recordings were done using standard two-electrode voltage clamp at room temperature. The oocytes were clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT, USA) was perfused with ND96 at 4-6 mL/min. Solutions were gravity-applied from 30-ml glass syringes with glass luer slips via Teflon tubing and switched manually. Voltage-recording and current-injecting electrodes were pulled from G120F-4 borosilicate glass (Warner Instruments). When filled with 3 M KCl the pipette resistances were 0.3-1 MΩ. The current
responses were amplified with an Axoclamp 900A (Molecular Devices, San Jose, CA, USA) or OC-725C amplifier (Warner Instruments), digitized with a Digidata 1320 or 1200 series digitizer (Molecular Devices), and stored on PC hard drive using pClamp (Molecular Devices). The current traces were analyzed using Clampfit (Molecular Devices) to determine current amplitude.

The effects of the modulators were estimated by comparing amplitudes of responses to GABA in the presence and absence of a modulator. The experiments were done as "continuous applications" where a cell is first exposed to GABA for up to several minutes, followed without washout by exposure to GABA + modulator, or as standard, separate applications where exposures to GABA and GABA + modulator are separated by washouts (>2 min) in bath solution. In potentiation concentration-response experiments, each cell was exposed to the full range of potentiator concentrations. In inhibition measurements, each cell was exposed to a single application of a single concentration of the inhibitor, and the concentration-response relationship was determined from pooled data. Concentration-response relationships were analyzed by fitting the curves to the Hill equation. Mechanistic analysis of modulatory effects of the DM compounds was done in the framework of the two-state (Resting-Active) concerted transition model as described in detail previously (Akk et al., 2018; Steinbach and Akk, 2019). The peak responses to GABA or GABA + modulator were converted to units of probability of being in the active state (P_A units) by normalizing the responses to the peak response to 1 mM GABA + 50 µM propofol in the same cell (Eaton et al., 2016). The latter was considered to have a peak P_A indistinguishable from 1 (Shin et al., 2017). The P_A data were fitted to the state function:

$$P_A = \frac{1}{1 + L^* \cdot \left( \frac{1 + [\text{modulator}]/K_{R,\text{modulator}}}{1 + [\text{modulator}]/(K_{R,\text{modulator}} \cdot c_{\text{modulator}})} \right)^{N_{\text{modulator}}}}$$

where $L^*$ expresses the $P_A$ of activity in the presence of low GABA alone and is calculated as $(1 - P_{A,\text{GABA}})/P_{A,\text{GABA}}$. $K_R$ is the equilibrium dissociation constant for the modulator in the resting receptor, $c$ is the ratio of the equilibrium dissociation constant in the active receptor to $K_R$, [modulator] is the
concentration of a DM compound, and N is the number of DM binding sites (constrained to two).

Curve-fitting was done using Origin 2020 (OriginLab Corp, Northampton, MA, USA).

The extent of modulation, $K_R$, and $c$ values are reported as mean ± S.D. Sensitivity to potentiator (EC$_{50}$) is reported as geometric mean with 95% confidence interval. Sensitivity to inhibitor (IC$_{50}$) was estimated from pooled data and is reported as best-fit parameter ± standard error of the fit.

**Molecular docking and molecular dynamics studies on the GABA$_A$R model**

The molecular model of the rat α1β2γ2 GABA$_A$R was constructed using the structure of the human α1β2γ2 GABA$_A$R [PDB: 6X3T; (Kim et al., 2020)] as a homolog template via the Prime module of the Schrödinger Suite Release 2020-3 (Schrödinger, LLC, New York, NY, USA). For molecular docking studies, the structures of the DM compounds and PAM-4 were prepared using 2D Sketcher and LigPrep within Schrödinger Maestro (Maestro Version 12.5.139) and evaluated for their ionization states at pH 7.4. Each ligand was docked to different subunit interfaces (i.e., β/α, α/β, α/γ, and γ/β) and ion channel of the GABA$_A$R model using QuickVina-W (Hassan et al., 2017), with the exhaustiveness parameter set to 300. Ten docking runs were performed under the same conditions, each producing twenty poses. The poses with more negative theoretical binding energy (TBE) values, obtained as described below, indicating higher theoretical binding affinities, were kept.

To maximize the conformer searching, the extracellular (ECD) and transmembrane (TMD) domains were also targeted separately. From a total of 200 conformers for each ligand, only those with root mean square deviation (RMSD) values (i.e., inter-molecular conformational changes, and the rotation and translation of the whole molecule) above two, were kept. The accepted conformers were clustered, using the same RMSD values as similarity scores, to define structural binding sites. The core of the QuickVina-W algorithm always implements rotations in every atom-atom rotatable bond of the ligand. However, as we are implementing “blind” docking, receptor residues are kept fixed at their original positions. To implement rotations to the receptor residues, a specific list of
rotatable bonds needs to be given, which is impracticable in blind docking. Usually, this is
implemented for docking to well-known binding pockets. To estimate accurate ligand TBEs, the
rescoring protocol MM-GBSA (Wang et al., 2019) in Schrodinger Prime (Schrödinger, 2020) was
used instead scoring functions as those found in QuickVina-W. This procedure allowed us to select,
from the initial 600 conformers, the conformer with the best theoretical binding affinity (i.e., more
negative TBE values), at each binding site. MM-GBSA energies, are thus used to rank the hundreds
of initial poses for each compound at every docking site, with more accurate values than those by the
docking method “scores”, however, they cannot be translated directly to K_D values. For this aim, i.e.
to obtain “absolute” binding energies, more sophisticated and time consuming approaches are
needed, such as alchemical methods (Williams-Noonan et al., 2018), but this is impracticable for the
number of poses we need to analyze.

The final selected docked conformers were further analyzed using molecular dynamics (MDs) to
determine their stability and behavior within the binding site. Here, the ligands and the whole
receptor are free to move during the MD. First, membrane building (dipalmitoylphosphatidylcholine
and cholesterol), complex solvation and ionization were carried out using the CHARMM-GUI
membrane builder input generator (Wu et al., 2014). The system, consisting of a hexagonal box of
X=92Å, Y=92Å, and Z=120Å dimension, 70 dipalmitoylphosphatidylcholine molecules, and 9
cholesterol molecules in each leaflet, was neutralized with 0.15 M KCl. To determine the stability of
the selected poses within their predicted docking sites, MD simulations of 100 ns were performed
using the program NAMD, CHARMM force field (Huang et al., 2017), and the CHARMM-GUI
NAMD input generator. Energy minimization of the complexes was first performed, followed by
several equilibration MDs (a total of 12 ns for the equilibration), where decreasing harmonic
restraints were applied to the backbone atoms. Finally, 100 ns production MDs were performed with
no restrictions, using a canonical NPT ensemble and a Nose-Hoover Langevin piston pressure
control, at 298 K. The MD protocol includes a timestep size of 2 fs, with 20 timesteps per cycle (the
number of timesteps between atom reassignments). The cutoff value for non-bond energy evaluation
was 10 Å. A distance of 8 Å for the switching function was used. Pairs of bonded atoms excluded from non-bonded interaction calculations was determined to 1-4, that is, no non-bonded interactions were calculated for lists of 4 consecutive bonded atoms. RMSD values for each ligand were extracted every 10-ps from the MD simulations using the VMD (Humphrey et al., 1996) RMSD Visualizer Tool plugin. Poses with RMSD variance (VAR) < 0.2 during the last third of the MD were considered stable, those with RMSD VAR values between 0.2 and 2 were considered nearly stable, and those with RMSD VAR values >2 were considered unstable. We also considered stable ligand-residue interactions that maintain, for at least 99% of the MD simulation, a distance ≤5 Å between the closest atoms and a distance ≤7 Å between the mass centers. MD simulations were performed using the PETE supercomputer at the High Performance Computing Center (Oklahoma State University-Center for Health Sciences, Tulsa, OK).
Results

Chemical synthesis and chemical characterization of the novel DM compounds

The method previously described for the synthesis of PAM-2 and DM489 (Arias et al., 2020) was applied to the synthesis of novel compounds. For DM497, (E)-3-(2-thienyl)acrylic acid was coupled with p-toluidine, using EDC and HOBT as coupling reagents. For DM492, DM495 and DM480, and PAM-4, (E)-3-(2-furanyl)acrylic acid was coupled with tetrahydroquinoline, tetrahydroisoquinoline, 1-aminoindoline hydrochloride or aniline, using EDC and HOBT as coupling reagents. (Fig. 1A). For the synthesis of DM490, PAM-2, used as a starting point, was deprotonated with sodium hydride and methylated using iodomethane (Fig. 1B). All compounds are >95% pure as determined by HPLC/diode-array detection (DAD) analysis. The chemical characteristics of PAM-2, DM489, and PAM-4 have been reported earlier (Arias et al., 2020; Arias et al., 2011). The chemical characterization of the DM compounds gave the following parameters:

DM497 [(E)-3-(thiophen-2-yl)-N-(p-tolyl)acrylamide]: $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 2.32 (s, 3H, CH$_3$), 6.34 (d, J = 15.2 Hz, 1H), 7.04 (dd, J=5.1, 3.7 Hz, 1H), 7.13 (d, J = 8.1 Hz, 2H), 7.22-7.24 (m, 1H), 7.25 (s, 1H), 7.32 (d, J = 5.1 Hz, 1H), 7.45-7.52 (m, 2H, NH+ 1H), 7.85 (d, J = 15.2 Hz, 1H) ppm. $^{13}$C NMR (CDCl$_3$, 400 MHz) $\delta$ 21.1, 120.53, 125.31, 127.61, 128.02, 130.23, 130.42, 134.02, 134.42, 135.71, 140.0, 164.5 ppm. ESI-LC/MS (C$_{14}$H$_{13}$NOS): m/z 244.3 [M+H]$^+$; mp 141-142 °C.

DM490 [(E)-3-(furan-2-yl)-N-methyl-N-(p-tolyl)acrylamide]: $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 2.39 (s, 3H, CH$_3$), 3.34 (s, 3H, NCH$_3$), 6.26 (d, J = 15.2 Hz, 1H), 6.36-6.38 (m, 1H), 6.47 (d, J=3.2 Hz, 1H), 7.08 (d, J = 8.0 Hz, 2H), 7.24 (d, J=12.8 Hz, 2H), 7.31 (s, 1H), 7.42 (d, J = 15.2 Hz, 1H) ppm. $^{13}$C NMR (CDCl$_3$, 400 MHz) $\delta$ 20.9, 37.5, 111.9, 113.5, 116.4, 126.9, 128.2, 130.1, 137.3, 140.9, 143.7, 151.6, 165.9 ppm. ESI-LC/MS (C$_{15}$H$_{15}$NO$_2$): m/z 242.2 [M+H]$^+$; mp 108-109 ºC.

DM492 [(E)-1-(3,4-dihydroquinolin-1(2H)-yl)-3-(furan-2-yl)prop-2-en-1-one]: $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 1.94-2.01 (m, 2H, CH$_2$), 2.72 (t, J=6.5 Hz, 2H, CH$_2$), 3.88 (t, J=6.6 Hz, 2H, CH$_2$), 6.40-6.42 (m, 1H), 6.52 (d, J=3.2 Hz, 1H), 6.74 (d, J=15.2 Hz, 1H), 7.12-7.37 (m, 4H), 7.37 (s, 1H), 7.50
(d, J = 15.2 Hz, 1H) ppm. $^{13}$C NMR (CDCl$_3$, 400 MHz) δ 24.10, 26.98, 43.38, 112.14, 113.72, 117.70, 124.99, 125.24, 126.21, 128.41, 128.80, 133.23, 138.57, 144.00, 151.78, 166.02 ppm. ESI-LC/MS (C$_{16}$H$_{15}$NO$_2$): m/z 254.3[M+H]$^+$; mp 119-120 °C.

DM495 [(E)-1-(3,4-dihydroisoquinolin-2(1H)-yl)-3-(furan-2-yl)prop-2-en-1-one]: $^1$H NMR (CDCl$_3$, 400 MHz) δ 2.89-2.93 (m, 2H, CH$_2$), 3.85-3.90 (m, 2H, CH$_2$), 4.82 (s, 2H, CH$_2$), 6.42-6.46 (m, 1H), 6.55 (d, J=2.8 Hz, 1H), 6.86 (d, J=15.2 Hz, 1H), 7.18-7.27 (m, 4H), 7.45 (s, 1H), 7.49 (d, J = 15.2 Hz, 1H) ppm. $^{13}$C NMR (CDCl$_3$, 400 MHz) δ 29.68, 40.30, 42.61, 112.14, 114.03, 114.79, 126.15, 126.84, 128.25, 128.61, 129.29, 142.94, 151.15, 166.10 ppm. ESI-LC/MS (C$_{16}$H$_{15}$NO$_2$): m/z 254.3[M+H]$^+$; mp 75-78 °C.

DM480 [(E)-3-(furan-2-yl)-N-(indolin-1-yl)acrylamide]: $^1$H NMR (CDCl$_3$, 400 MHz) δ 3.02-3.12 (m, 3H, CH), 3.61-3.69 (m, 1H, CH), 3.88 (t, J=6.6 Hz, 2H, CH$_2$), 6.40-6.42 (m, 1H), 6.52 (d, J=3.2 Hz, 1H), 6.74 (d, J=15.2 Hz, 1H), 7.12-7.37 (m, 4H), 7.42 (s, 1H), 7.56 (d, J = 15.2 Hz, 1H) ppm. $^{13}$C NMR (CDCl$_3$, 400 MHz) δ 24.10, 26.98, 43.38, 112.14, 113.72, 117.70, 124.99, 125.24, 126.21, 128.41, 128.80, 133.23, 138.57, 144.00, 151.78, 166.02 ppm. ESI-LC/MS (C$_{15}$H$_{14}$N$_2$O$_2$): m/z 254.3[M+H]$^+$; mp 125-128 °C.

The novel DM compounds increase the apparent affinity of GABA in the $\alpha_1\beta_3\gamma_2$L GABA$_A$R

The effect of each DM compound on the apparent binding affinity of GABA at $\alpha_1\beta_3\gamma_2$L GABA$_A$Rs was determined by monitoring GABA$_A$R-bound Gzn-OG fluorescence at varying concentrations of GABA in the absence and presence of 10-100 µM of each DM compound (Fig. 2). Increasing concentrations of GABA decreased $\alpha_1\beta_3\gamma_2$L-bound Gzn-OG fluorescence, making it nearly undetectable at 1 mM GABA. The inhibition curves were shifted to lower GABA concentrations in the presence of the DM compounds, although the magnitudes of effects differed for individual compounds (Fig. 3). For example, lower Gzn-OG fluorescence was observed in the presence of DM490 compared to DM480, suggesting that the former drug is more efficient in increasing displacement of Gzn-OG by GABA.
Fitting the F/F₀ curves to Eq. 1 yielded IC₅₀ values that were transformed to apparent Kₐ,GABA using Eq. 2. To assess the variability between GABA titrations, the Kₐ,GABA were calculated on a daily basis, rendering values between 13.0 ± 1.1 µM and 19.0 ± 1.1 µM (mean ± SEM; n = 15-22 cells/titration) (Table 2). In the presence of increasing concentrations of each DM compound, the GABA concentration-response curves were shifted to lower transmitter concentrations (Fig. 3). Considering the Kₐ,GABA shift between 0 (control) and 100 µM DM compound (i.e., Kₐ,GABA/Kₐ,GABA,DM), two populations were discriminated: compounds that induced ≥60-fold decrease in Kₐ,GABA (i.e., DM497, DM490, and DM492), and compounds that produced ≤9-fold change (Table 2). Thus, all tested DM compounds increased the apparent binding affinity of GABA, while DM497, DM490, and DM492 produced the largest effects. DM480 was largely ineffective (Fig. 3).

We also assessed whether the most potent DM compounds directly affect the interaction of Gzn-OG with the orthosteric site at GABAₐRs (Fig. 3G-H). These results indicated that neither DM497 nor DM490 modifies α1β3γ2L-bound Gzn-OG. This suggests that the novel compounds allosterically increase the apparent affinity of GABA to the α1β3γ2L GABAₐR rather than directly or indirectly modify the interaction of Gzn-OG with orthosteric binding sites.

Potentiating effects of the novel DM compounds on α₁β₂γ₂L and α₁β₃γ₂L GABAₐRs

Next, we employed two-electrode voltage-clamp electrophysiology to assess the activity of the novel DM compounds and PAM-4 on the synaptic-type α₁β₂γ₂L GABAₐR. To better align the electrophysiological data to fluorescence measurements, selected experiments were additionally conducted on the α₁β₃γ₂L subtype. The overall findings are that DM489, DM490, DM492, DM495 and DM497 potentiate currents elicited by low GABA and that the modulatory effects of the compounds are similar in α₁β₂γ₂L and α₁β₃γ₂L receptors. Due to the small effect on fluorescence elicited by DM480, this compound was not studied in electrophysiological recordings.

The concentration-response relationships for DM497, DM492, and DM490 were measured by
coapplying 1-100 µM of each compound with low (3-10 µM; PA<0.20) GABA. Fitting the concentration-response curves to the Hill equation gave the EC_{50} values (geometric means with 95% confidence intervals) of 32 µM [20 to 50 µM] for DM497, 15 µM [11 to 19 µM] for DM492, and 12 µM [10 to 15 µM] for DM490. Sample current traces and the concentration-response relationships are shown in Figure 4 and summarized in Table 3. DM489 and DM495 were relatively weak modulators, accordingly full concentration-response relationships were not measured. Both compounds were without effect on low-GABA currents at 10 µM. At 50 µM, DM489 and DM495 potentiated the responses to GABA to 144 ± 9% (n = 5) or 189 ± 53% (n = 5) of control, respectively.

The potentiating effects of DM497, DM492 and DM490 were further analyzed in the framework of the two-state (Resting-Active) co-agonist model (Steinbach and Akk, 2019). Fitting the concentration-response data to Eq. 3 yielded K_R (the equilibrium dissociation constant for the modulator in the resting receptor) values of 50 ± 33 µM, 19 ± 7 µM and 22 ± 3 µM, and c (the ratio of the equilibrium dissociation constant in the active receptor to K_R) values of 0.391 ± 0.157, 0.409 ± 0.157 and 0.224 ± 0.048 for DM497, DM492 and DM490, respectively (Table 3). The calculated free energy change [ΔG=NRTln(c)] to stabilize the active state ranges from -1.13 kcal/mol (DM492) to -1.79 kcal/mol (DM490) (Table 3). To put this in wider perspective, the ΔG values are similar to those of the neurosteroid allopregnanolone and the benzodiazepine diazepam (Cao et al., 2018; Shin et al., 2019), and the previously-reported structural analog of the DM compounds, PAM-2 (Arias et al., 2022).

To better link electrophysiology to fluorescence data, additional experiments were conducted on the α1β3γ2L receptor. The receptor was activated by a low concentration (1-2 µM; <EC_{15}) of GABA, and the modulatory effects of each compound measured. The DM compounds were tested at concentrations (50 µM) that produced a near-saturating effect in the α1β2γ2L receptor. The main finding is that the magnitudes of potentiating effects of the DM compounds are statistically (t-test; Excel, Microsoft, Redmond, WA) indistinguishable in α1β2γ2L and α1β3γ2L receptors. The data are
summarized in Figure 5.

**Inhibitory effects of PAM-4 and the novel DM compounds on the α1β2γ2L GABA<sub>A</sub>R**

PAM-4 inhibited responses to low GABA. Coapplication of 1-500 µM PAM-4 reduced the responses to 2-5 µM GABA (<P<0.10) to ~50% of control, with the IC<sub>50</sub> at 54 ± 7 µM (best-fit parameter ± standard error of the fit of pooled data, n = 5-11 oocytes per concentration). Sample current traces and the concentration-response curve are given in Figure 6.

To determine if inhibition by PAM-4 and potentiation by DM497 is mediated by same or distinct sites, we measured and analyzed potentiation of GABA-activated receptors by DM497 in the presence of a fixed concentration of PAM-4. We reasoned that if PAM-4 acts independently of DM497 then its effect manifests as reduced current response both in the absence and presence of DM497. In this model, the fitted affinity and efficacy parameters describing receptor potentiation by DM497 are expected to be indistinguishable from those estimated in the absence of PAM-4. In contrast, if PAM-4 inhibits and DM497 potentiates the receptor through the same set of binding sites then the inhibitory effect of PAM-4 is expected to manifest as reduced apparent affinity of DM497 (increased K<sub>R,DM497</sub>) but enhanced apparent efficacy (reduced apparent c<sub>DM497</sub>) because at high concentrations DM497 outcompetes PAM-4 and the extent of potentiation reaches the control level.

We recorded GABA-elicited currents in the presence of 1-100 µM DM497 coapplied with 50 µM or 200 µM PAM-4 (Fig. 6B). Coapplication of 50 µM PAM-4 reduced the P<sub>A</sub> of the response to 2 µM GABA from 0.057 ± 0.011 to 0.041 ± 0.011 (n = 6). Exposure to 100 µM DM497 enhanced the response to GABA + PAM-4 to 0.122 ± 0.03. The EC<sub>50</sub> of the potentiation curve was 26 µM [23 to 30 µM] (Fig. 6C), which is not significantly different from the EC<sub>50</sub> for potentiation by DM497 in the absence of PAM-4 (32 µM, see above). Fitting of the potentiation curve to Eq. 3 yielded a K<sub>R,DM497</sub> of 29 ± 9 µM and a c<sub>DM497</sub> of 0.454 ± 0.031. Neither value is significantly different from the estimates in the absence of PAM-4. In the presence of 200 µM PAM-4, the EC<sub>50</sub> of the DM497 potentiation curve was 28 µM [24 to 33 µM], and the fitted K<sub>R,DM497</sub> and c<sub>DM497</sub> 43 ± 21 µM and
0.618 ± 0.036, respectively. The EC50 and K_{R,DM497} estimates are not different from those in the absence of PAM-4, but c_{DM497} is significantly (P < 0.05) higher than that in the absence of PAM-4. The reason for higher c_{DM497} (i.e., reduced apparent efficacy) in the presence of 200 µM PAM-4 is unclear, however we note that such an effect is the opposite to what is expected if PAM-4 and DM497 bind to overlapping sites. In sum, we infer that GABA_{A}R inhibition by PAM-4 and potentiation by DM497 are mediated by distinct sites.

State-dependence of inhibition and distinct mechanisms of inhibition manifest in dissimilar sensitivity to inhibitor at different levels of activity (Germann et al., 2022). To gain insight into the mechanism of inhibition by PAM-4, we compared the magnitude of inhibition at low (2-5 µM; peak P_{A}=0.1) and high (1 mM; peak P_{A}=0.9) GABA. Application of 50 or 200 µM PAM-4 reduced the response to low GABA to 81 ± 8% (n = 5) or 48 ± 16% (n = 11) of control, and the response to high GABA to 84 ± 7% (n = 5) or 58 ± 11% (n = 5) of control, respectively. The magnitudes of effects are statistically indistinguishable. We infer that PAM-4 similarly inhibits resting, active, and desensitized receptors.

The α1(V256S) mutation abolishes inhibition by PAM-4 and the novel DM compounds

Under recording conditions where potentiation dominates, i.e., at low fractional activities, small inhibitory effects can be masked by a separate, stronger potentiating effect. To test if the DM compounds can inhibit the GABA_{A}R, we measured the effect of each DM compound (DM490, DM497, DM492, DM489, DM495) on steady-state current elicited by 1 mM GABA. At this concentration of agonist, potentiating effects of a drug are masked due to the high P_{A} (0.9) of the current response (Shin et al., 2017; Szabo et al., 2019). The main finding is that all compounds inhibited responses to GABA. The effects among the compounds, applied at 50 µM, were relatively similar with the magnitude of inhibition ranging from 33 ± 7% (n = 5; DM489) to 46 ± 15% (n = 5; DM495). The data are summarized in Figure 7A-B.

The α1(V256S) mutation (2' residue in TM2) drastically reduces the actions of inhibitory
neurosteroids and analogs (Akk et al., 2001; Li et al., 2006b; Wang et al., 2002). To probe if receptor inhibition by PAM-4 and the DM compounds involves the mechanism utilized by inhibitory neurosteroids, we measured inhibition of the α1(V256S)β2γ2L GABAAR by PAM-4 and the DM compounds. The receptors were initially exposed to 1 mM GABA ($P_{A,\text{peak}} = 0.87 \pm 0.12$, $n = 28$). At approx. 4-min time point, the solution was switched to GABA + 50 µM modulator, followed by wash in GABA. The main finding is that in the mutant receptor, coapplication of a modulator had minimal (<2%) inhibitory effect on the response to GABA. The data are summarized in Figure 7A-B.

In control experiments, we tested DM-mediated potentiation of the α1(V256S)β2γ2L receptor activated by low (0.05 µM; $P_A = 0.07 \pm 0.05$, $n = 26$) GABA. The mutation did not reduce the potentiating effects of the DM compounds (Fig. 7C-D). Interestingly, we observed a small (109 ± 2% of control; $n = 5$) but statistically significant ($P < 0.001$ vs. no effect, paired t-test; Excel, Microsoft) potentiating effect in the presence of 100 µM PAM-4. We infer that the α1(V256S) mutation selectively reduces the inhibitory effects of PAM-4 and the DM compounds. From these data, we propose that all tested compounds have potentiating and inhibitory actions on the α1β2γ2L GABAAR while the magnitude and direction of the observed effect depend on recording conditions (low vs. high GABA, presence of the V256S mutation) that may selectively mask potentiation or inhibition.

**DM490 and DM497 stably dock to the anesthetic sites, whereas PAM-4 also docks to the ion channel lumen of the α1β2γ2 model**

Initial molecular docking results indicated that DM490, DM497, and PAM-4 interact with the classic anesthetic binding sites located at the intersubunit interfaces of the α1β2γ2 model (Fig. 8A), with binding energies in the -30 to -40 kcal/mol range (Table 4). DM497 and PAM-4 also interacted with the homologous site at the α+/γ− interface, i.e., at the interface formed by the "+" side of the α subunit and the "−" side of the γ subunit (Fig. 8A, middle panel) and ion channel (Fig. 8A, right panel), respectively. To determine ligand stability in each docking site, RMSD variance (VAR)
values during the last third of the MD simulations (100 ns) were calculated. Table 4 shows the theoretical binding energies (TBE) of the poses, along with their RMSD and RMSD VAR values, and the stability classification based on the latter (defined as “stable,” “near stable,” and “unstable”), as explained in Methods. The residue interactions of the stable poses are shown in Table 5. The docking of DM490, DM497, and PAM-4 at the β+/α− site were considered stable. Each ligand made contact with practically the same residues: β-TM2 (M261, T262, and N265) and β-TM3 residues (L285, M286, F289, and V290) (Table 5). In particular, DM490 established a π-π interaction with F289. At the α+/β− site, only DM490 remained stable (Table 4), making contacts with α-TM2 (T267 and S270), α-TM3 (A283, D287, W288, I290, A291, and Y294), and β-TM1 residues (L223, M227, P228, and L231) (Table 5). DM490 also established a π-π interaction with αY294. Figures 9A-B show the molecular interactions for DM490 at the respective β+/α− and α+/β− site.

DM497 was the only compound forming stable interactions with the anesthetic γ+/β− and α+/γ− sites. In the γ+/β− site, DM497 made contact with γ-TM2 (T277, S280; M3 S301, F304, I305, and F308) and β-TM1 residues (L223, Q224, M227, P228, and L231) (Table 5). In the α+/γ− site, DM497 interacted with α-TM2 (S270, M3 D287, I290, A291, Y294, A295, F298, S299, and I302) and α-TM1 residues (I238, I242, and L246) (Table 5). Figures 9C-D show the molecular interactions for DM497 at the β+/α− and α+/γ− sites.

PAM-4 was the only compound showing high stability (VAR = 0.06) in the ion channel site (Table 4), making contact with the α, β, and γ subunits (Fig. 9E). PAM-4 directly interacted with the ion channel lumen, more specifically with TM2 rings located at 6’ (α-T261), 9’ (γ-L274 and β-L259), and 13’ (γ-T278 and β-T263) positions, in addition to other non-luminal residues (Table 5). The differences in the binding interfaces may account for the differences in efficacies among the DM compounds and for the inhibitory activity of PAM-4. Interestingly, although DM490 is the most potent PAM using low GABA concentrations, it inhibits the receptor at high concentrations which we account for by its detection at the site in the channel (Table 4). At this site, DM490 interacts with the α (M1 P233, T237; M2 T261[6’], L264[9’], T265, M266, T268[13’], and L269) and β (V258,
L259[9’], T260, M261, T262, and T263[13’]) residues.

**Functional insights into potentiating sites**

A combination of agonists acting independently through distinct sites generates a larger potentiating effect and response than a combination of agonists that acts through same or overlapping sites (Shin et al., 2019). Molecular docking studies suggested that the DM compounds interact with the anesthetic binding sites in intersubunit interfaces. To confirm this functionally, we compared the potentiating effect of DM490 in receptors activated by GABA and in receptors activated by the anesthetics etomidate or phenobarbital. Etomidate and phenobarbital activate the GABA<sub>AR</sub> by interacting with the anesthetic binding sites in the β+/α−, and α+/β− and γ+/β− interfaces, respectively (Chiara et al., 2013; Kim et al., 2020; Li et al., 2006a). We reasoned that if DM490 potentiates the α1β2γ2L receptor by interacting with one or more of the anesthetic sites, then its effect on receptors activated by etomidate (or phenobarbital) is smaller than that in receptors activated by GABA. In contrast, if DM490 and etomidate (or phenobarbital) act through non-overlapping sites, and independently, then potentiation is expected to be similar in receptors activated by GABA or the anesthetics. Receptor responses to 5-10 µM etomidate (P<sub>A</sub> = 0.09 ± 0.05, n = 8) were only weakly (156 ± 60% of control) enhanced by 50 µM DM490. Coapplication of 50 µM DM490 potentiated the response to 1 mM phenobarbital (P<sub>A</sub> = 0.09 ± 0.04, n = 5) to 240 ± 67% of control. For comparison, DM490 potentiated receptors activated by 1 µM GABA to 887 ± 249% of control (above, Fig. 7D). Sample currents and the summary of potentiation of etomidate- and phenobarbital-activated receptors by DM490 are given in Figure 10A. We propose that GABA<sub>AR</sub> potentiation by DM490 utilizes the classic intersubunit anesthetic binding sites, with a possibly larger functional contribution made by the etomidate-binding β+/α− interfaces compared to the phenobarbital-binding α+/β− and γ+/β− interfaces.

Further tests of the involvement of the anesthetic sites in potentiation by the DM compounds were done on receptors containing mutations to one or more intersubunit interfaces. We first tested
the effect of the β2(F289A) mutation. This residue is in the β+/α− interface where it lines the binding pocket for etomidate and propofol (Chiara et al., 2012; Franks, 2015). Our molecular docking studies place β2(F289) within 4 Å of the DM490 molecule where it makes a π-π interaction with the phenyl group of DM490 (Fig. 8B). In the α1β2(F289A)γ2L receptor, coapplication of 50 µM DM490 potentiated the response to 0.1-0.2 µM GABA (P_A = 0.07 ± 0.05, n = 5) to a mere 114 ± 6% of control (vs. 887% of control in wild-type; see above). From P_A,50 µM DM490 = 1/(1 + L × c_{50 µM DM490}^2), we estimate a c_{50 µM DM490} of 0.935 ± 0.027 and a ΔG of -0.08 ± 0.03 kcal/mol. For comparison, the calculated c_{50 µM DM490} in the α1β2γ2L receptor is 0.296 ± 0.048 and the ΔG is -1.45 ± 0.19 kcal/mol.

Second, we measured the effect of the α1(Y293C) mutation on modulation by DM490. This residue, predicted to make π-π interaction with the phenyl group of DM490, is present in the α+/β− interface at the barbiturate binding site (Chiara et al., 2013; Kim et al., 2020) and in the α+/γ− interface, termed the "orphan" interface due to lack of protection by the anesthetics propofol, etomidate or a barbiturate analog in cysteine-modification experiments (Nourmahnad et al., 2016). We note that the rat α1(Y293) residue is homologous to the human α1(Y294) residue in modelling studies (Fig. 9). In the α1(Y293C)β2γ2L receptor, coapplication of 50 µM DM490 potentiated the response to 0.1-0.2 µM GABA (P_A = 0.05 ± 0.04, n = 5) to 891 ± 353% of control (Fig. 10C). The magnitude of potentiation was indistinguishable from potentiation observed in the α1β2γ2L receptor. The calculated c_{50 µM DM490} is 0.293 ± 0.041 and the ΔG is -1.46 ± 0.17 kcal/mol.

We also measured the effect of γ2L(L246W) (10' position after the conserved GYF sequence in TM1). This mutation is present solely in the α+/γ− interface. The α1β2γ2L(L246W) receptor is constitutively active (P_{A,constitutive} = 0.12 ± 0.04; n = 5); accordingly, we measured the direct activating, rather than potentiating, effect of DM490. Application of 50 µM DM490 generated a response with a P_A of 0.31 ± 0.07 (Fig. 10C). The calculated c_{50 µM DM490} is 0.537 ± 0.032 and the ΔG is -0.74 ± 0.07 kcal/mol.

In the barbiturate-binding γ+/β− interface, we tested the γ2L(F304C) mutation. Since the α1β2γ2L(F304C) receptor is constitutively active (P_{A,constitutive} = 0.32 ± 0.10; n = 5), the effect of
DM490 was tested in the absence of GABA. Application of 50 µM DM490 increased the $P_A$ to 0.46 ± 0.11 (1.5 ± 0.2-fold potentiation in $P_A$ units). The calculated $c_{50\mu M\text{DM490}}$ and $\Delta G$ are 0.736 ± 0.035 and -0.36 ± 0.06 kcal/mol, respectively. The findings on the mutant receptors are summarized in Figure 10D.

In sum, two sets of experiments indicate that the potentiating action of DM490 is mediated by the classic anesthetic binding sites at intersubunit interfaces in the transmembrane domain of the receptor. First, DM490 has a weaker potentiating effect on receptors activated by the anesthetics etomidate or phenobarbital compared to receptors activated by GABA. Second, mutations to the individual intersubunit interfaces, in some cases drastically, reduce the potentiating effect of DM490. We note that with a single exception (the $\alpha_1(Y293C)$ mutation to the $\alpha^+\beta^-$ interface), the predictions from molecular docking were confirmed in electrophysiological studies.

**Functional insights into inhibitory sites**

Sensitivity to the $\alpha_1(V256S)$ mutation suggests that a mechanism similar to that of inhibitory neurosteroids and analogs (Akk et al., 2001; Li et al., 2006b; Wang et al., 2002) underlies inhibition by PAM-4 and the DM compounds. We emphasize that at this time it is unclear if the $\alpha_1V256$ residue is a component of the binding site or a transduction element for inhibitory steroids (Akk et al., 2001). We next investigated whether PAM-4 and the inhibitory steroid pregnenolone sulfate (PS) interact with overlapping binding sites. We recorded the effect of 200 µM PAM-4 on steady-state current generated by 1 mM GABA in the presence of 10 µM PS. PAM-4 (Fig. 6C) is a lower efficacy inhibitor than PS (Fig. 11A; (Germann et al., 2019; Pierce et al., 2022)). We hypothesized that interaction with overlapping sites would lead to competition with PS-binding and manifest as reduced PS-inhibition in the presence of PAM-4. Application of 10 µM PS reduced the steady-state response to 1 mM GABA to 16 ± 4% (n = 5) of control. Coapplication of 200 µM PAM-4 with GABA + PS increased inhibition, reducing the steady-state response to 11 ± 3% (n = 5) of the control GABA steady-state response (or 67 ± 9% of the response to GABA + PS). A sample current
trace is given in Figure 11A. Given increased inhibition when PAM-4 is coapplied with PS, we infer that PAM-4 does not interfere with PS binding to its site.

An analogous experiment was conducted using DM490 as the competing agent. The receptors were activated with 1 mM GABA. Once steady-state response was reached, the cells were exposed to GABA + 10 µM PS, followed by an application of GABA + PS + 50 µM DM490. In five cells, the application of PS reduced the steady-state response to 12 ± 5% of the GABA control response whereas the application of DM490 reduced the response to 6 ± 2% of the GABA control response (or 55 ± 6% of the GABA + PS response). The effect of 50 µM DM490 is similar in the absence (Table 3) and presence of PS. We conclude that PS does not interfere with DM490-elicited inhibition and propose that DM490 and PS bind to distinct sites.

Molecular docking suggested a possible binding site for PAM-4 in the channel lumen where it interacts with residues in the 6', 9', and 13' rings of TM2 (Fig. 9E). To probe the functional involvement of this site, we measured the effects of 200 µM PAM-4 on steady-state current elicited by 1 mM GABA in α1β2γ2L(L274C) (9' leucine in γ-TM2) and α1β2γ2L(T278C) (13' threonine in γ-TM2) receptors. As control, we tested receptor inhibition by 10 µM PS. In the α1β2γ2L(L274C) receptor, application of PAM-4 reduced the PA of the steady-state current from 0.87 ± 0.04 (n = 5) to 0.82 ± 0.03 (6 ± 1% inhibition). This should be compared to the ~50% inhibition in α1β2γ2L (Fig. 6A, C). In a control experiment, PS fully abolished receptor activity: PA of the steady-state response decreased from 0.85 ± 0.07 (n = 5) to -0.18 ± 0.20. Sample current traces and data summary are given in Figure 11. In the α1β2γ2L(T278C) receptor (Fig. 11C), the PA of the steady-state current decreased from 0.20 ± 0.08 (n = 5) to 0.17 ± 0.06 (11 ± 11% inhibition) in the presence of PAM-4. In contrast, exposure to PS reduced the PA from 0.18 ± 0.07 to 0.02 ± 0.01 (90 ± 2% inhibition). Thus, mutations to selected pore-lining residues in the γ2L subunit strongly reduce receptor inhibition by PAM-4. The magnitudes of the inhibitory effects of PS in the mutant receptors are similar to those observed in the wild-type ternary αβγ receptor (Germann et al., 2019; Pierce et al., 2022) and support the notion that PS and PAM-4 bind to distinct sites. The data are summarized in
Figure 11D.

We conclude that PAM-4 and DM490 inhibit the receptor through interactions with a site that is distinct from that of the inhibitory steroid PS. We have also shown that substitutions of residues in the lumen of the channel (9' and 13' in TM2 of the γ2L subunit) strongly reduce inhibition by PAM-4 but not by PS.
Discussion

The objective of this study was to develop and characterize novel acrylamide-derived compounds, based on the structure of the previously-reported GABAergic drug PAM-2 (Arias et al., 2022), as modulators of the GABA<sub>A</sub>R. We show that the newly synthesized compounds, at high micromolar concentrations, concurrently act as allosteric potentiators and inhibitors of the ternary αβγ GABA<sub>A</sub>R. The relative magnitudes of the potentiating and inhibitory effects differ among the compounds thereby defining the experimentally observed net functional effect. For example, when tested in the presence of a low concentration of GABA, the strong potentiating effect of DM490 overwhelms its relatively weak inhibitory effect, whereas its much weaker potentiating effect renders PAM-4 a net inhibitory compound. When tested in the presence of saturating GABA, where any observable potentiation is diminished due to the high P<sub>A</sub> of the control response, all compounds produced inhibition.

The free energy changes provided by the most efficacious DM compounds to stabilize the active state (-1.13 to -1.79 kcal/mol for DM492, DM497 and DM490) are similar to those for several neurosteroids, the benzodiazepine diazepam, and the previously-studied acrylamide-based GABA<sub>A</sub>R potentiation PAM-2 (Arias et al., 2022; Cao et al., 2018; Shin et al., 2019). Such compounds do not meaningfully directly activate the wild-type α1β2γ2L GABA<sub>A</sub>R (for example, a ΔG of -1.4 kcal/mol predicts a P<sub>A,peak</sub> of 0.001), but efficaciously potentiate a receptor activated by low concentrations of GABA (a ΔG of -1.4 kcal/mol predicts a >5-fold potentiation of an EC<sub>10</sub> / P<sub>A</sub> 0.1 response). DM489 and DM495 were weaker modulators with ΔG values at -0.30 to -0.20 kcal/mol, and the potentiating effect of PAM-4 could only be observed when its inhibitory effect was blocked by a mutation.

The potentiating and inhibitory effects of the DM compounds and PAM-4 are mediated by distinct sites. Molecular docking analysis suggested that the DM compounds potentiate the receptor through interactions with the classic anesthetic binding sites in the intersubunit interfaces. In electrophysiological recordings, DM490 had a smaller potentiating effect on receptors activated by the allosteric agonists etomidate and phenobarbital compared to receptors activated by GABA. We
interpret this finding as DM490 sharing binding sites with etomidate and phenobarbital. From the relative effects on etomidate- vs. phenobarbital-activated receptors we propose that the interaction of DM490 with the two β+/α− interfaces makes a slightly larger energetic impact than its interaction with the phenobarbital-binding α+/β− and γ+/β− interfaces. We note though that the calculated contributions are not strictly additive. In α1β2γ2L receptors activated by GABA alone, in which all anesthetic sites are free to bind the modulator, 50 µM DM490 contributes -1.45 ± 0.19 kcal/mol free energy change towards activation. In etomidate-activated receptors, where the α+/β− and γ+/β− interfaces are presumed vacant, DM490 contributes -0.25 ± 0.23 kcal/mol, and in phenobarbital-activated receptors, DM490 binding to the β+/α− interfaces contributes -0.58 ± 0.18 kcal/mol. Thus, the loss of potentiation in receptors activated by etomidate or phenobarbital is greater than what is expected in case of independence of sites and energetic additivity. One conceivable explanation is that etomidate and/or phenobarbital interact with interfaces other than those postulated.

Lack of energetic additivity was also observed in experiments on receptors containing mutations to the individual anesthetic binding interfaces. Almost a complete loss of activity (ΔG50 µM DM490 = -0.08 ± 0.03 kcal/mol) was observed in the receptor containing the β2(F289A) mutation in the two β+/α− interfaces, suggesting that this interface is the major, nearly sole contributor to activation and potentiation by DM490. On the other hand, mutations to the barbiturate-binding γ+/β− and the orphan α+/γ− interfaces also significantly reduced gating by DM490. The free energy changes supplied by 50 µM DM490 were -0.36 ±0.06 kcal/mol (ΔΔG = 1.09 kcal/mol vs. wild-type) and -0.74 ± 0.07 kcal/mol (ΔΔG = 0.75 kcal/mol vs. wild-type) in the α1β2γ2L(F304C) and α1β2γ2L(L246W) receptors, respectively, overall suggesting strong contributions from interfaces other than β+/α−. The most parsimonious explanation is that the anesthetic binding sites in different intersubunit interfaces are allosterically linked as reported previously (Hoyt et al., 2022; Szabo et al., 2019). The putative binding site location is supported by molecular docking and molecular dynamics studies where DM490 stably interacted with the β+/α− and α+/β− interfaces, whereas DM497 also interacted with the γ+/β− and α+/γ− interfaces.
While receptor inhibition by PAM-4 and the DM compounds shows similarities to the actions of inhibitory neurosteroids, such as sensitivity to the α1(V256S) mutation, our functional competition experiments (Fig. 11A) indicate that PAM-4 or DM490, and PS do not share a binding site. This is supported by results on mutant receptors. From molecular docking, we have postulated a binding site for PAM-4 in the channel lumen near the activation gate at the TM2-9'-13' level where mutations to selected residues strongly diminished inhibition by PAM-4 but not by PS (Fig. 11D).

In sum, we report here the chemical synthesis and functional characterization of novel acrylamide-based modulators of the mammalian GABA<sub>A</sub> receptor. The compounds have concurrent potentiating and inhibitory effects with net positive modulatory efficacies reaching that of a number of GABAergic anxiolytics and sedatives. The synthesized compounds have medium to high blood-brain barrier penetration (Table 1), supportive of potential use in clinical practice.
Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper and its Supplemental Data.

Authorship Contributions

Participated in research design: Arias, Ortells, Sakamoto, and Akk.

Conducted experiments: Pierce, Germann, Xu, Ortells, and Sakamoto.

Contributed new reagents or analytic tools: Manetti, Romanelli, and Hamachi.

Performed data analysis: Arias, Pierce, Germann, Xu, Ortells, Sakamoto, and Akk.

Wrote or contributed to the writing of the manuscript: Arias, Pierce, Germann, Xu, Ortells, Sakamoto, Manetti, Romanelli, Hamachi, and Akk.
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Footnotes

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM140947]; IMI2 project NeuroDerisk (821528); Italian Ministry of Instruction, University and Research (MIUR), Italy; the Taylor Family Institute for Innovative Psychiatric Research; and by an OVPR Pilot/Seed Grant (Oklahoma State University Center for Health Sciences). No author has an actual or perceived conflict of interest with the contents of this article.
Legends for Figures

Figure 1. Chemical synthesis. (A) Synthesis of DM497 (a), DM492 (b), DM495 (c), DM480 (d), and PAM-4 (e). The suitable carboxylic acid was reacted with different amines in CH$_2$Cl$_2$ in the presence of Et$_3$N and using EDC and HOBt as coupling reagents. The respective amines were: p-toluidine (a), tetrahydroquinoline (b); tetrahydroisoquinoline (c), 1-aminindoline hydrochloride (d), and aniline (e). (B) Synthesis of DM490. The NH group of PAM-2 was first deprotonated using NaH in THF and then alkylated with MeI (f). More details are provided in Methods.

Figure 2. Fluorescence measurements. Confocal images of HEK293T α1β3γ2L cells upon the addition of the turn-on fluorescent probe Gzn-OG (100 nM), in the absence and presence of increasing concentrations (1 µM-10 mM) of GABA, administered alone or in the presence of 100 µM of each DM compound.

Figure 3. DM compounds modify apparent GABA binding affinity. (A-F) Fluorescence intensity of HEK293T-α1β3γ2L-bound Gzn-OG in the presence over absence (F/F₀) of 0.01 µM-30 mM GABA applied alone or in combination with 10, 30, or 100 µM DM497 (A), DM490 (B), DM492 (C), DM495 (D), DM489 (E), and DM480 (F). The data points show mean ± S.E.M. from 12 to 22 cells (see below for specifics). The GABA IC₅₀s were estimated from non-linear regression analysis of F/F₀ vs [GABA] relationships. The estimated IC₅₀s for the DM497 dataset are: 47.5 ± 4.6 µM (GABA alone, n = 22 cells), 5.0 ± 0.3 µM (10 µM DM497, n = 16), 2.2 ± 0.2 µM (30 µM DM497, n = 15), and 0.6 ± 0.4 µM (100 µM DM497, n = 16). The estimated IC₅₀s for the DM490 dataset are: 46.5 ± 3.3 µM (GABA alone, n = 15), 2.8 ± 0.2 µM (10 µM DM490, n =14), 2.1 ± 0.2 µM (30 µM DM490, n = 14), and 0.6 ± 0.1 µM (100 µM DM490, n = 14). The estimated IC₅₀s for the DM492 dataset are: 46.5 ± 3.3 µM (GABA alone, n = 15), 9.9 ± 0.5 µM (10 µM DM492, n = 15), 2.9 ± 0.4 µM (30 µM DM492, n = 16), and 0.7 ± 0.1 µM (100 µM DM492, n = 18). The estimated IC₅₀s for the DM495 dataset are: 36.6 ± 3.2 µM (GABA alone, n = 20), 29.3 ± 3.1 µM (10 µM DM495, n =
17), 18.4 ± 1.6 μM (30 μM DM495, n = 15), and 4.3 ± 0.3 μM (100 μM DM495, n = 15). The estimated IC₅₀s for the DM489 dataset are: 53.5 ± 3.2 μM (GABA alone, n = 16), 24.8 ± 2.0 μM (10 μM DM489, n = 16), 10.7 ± 0.7 μM (30 μM DM489, n = 21), and 5.9 ± 0.4 μM (100 μM DM489, n = 12). The estimated IC₅₀s for the DM480 dataset are: 36.5 ± 3.2 μM (GABA alone, n = 20), 24.6 ± 1.7 μM (10 μM DM480, n = 20), 14.9 ± 0.6 μM (30 μM DM480, n = 21), and 12.9 ± 1.1 μM (100 μM DM480, n = 16). The IC₅₀ values were transformed to apparent Kᵩ,GABA values using Eq. (2), and are provided in Table 2. (G) The effects of DM497 and DM490, as representative DM compounds, on α₁β₃γ₂L GABAₐR-bound Gzn-OG (n = 16 cells for each compound). The DM compounds did not displace Gzn-OG from the orthosteric binding site at concentrations up to 100 μM.

Figure 4. Electrophysiological activity of novel DM compounds and PAM-4 on the α₁β₂γ₂L GABAₐR activated by low GABA. Representative GABA (3-10 μM)-induced currents in the absence and presence of DM490 (A), DM497 (B), or DM492 (C). (D) Concentration-response relationships for DM490, DM497, and DM492. The data points show mean ± S.D. from 5 (DM490 and DM497) or 6 (DM492) oocytes. Each oocyte was exposed to the full range of drug concentrations. The fitted EC₅₀, E₅₀, and nᵣ values are summarized in Table 3.

Figure 5. Comparison of the effects of novel DM compounds on α₁β₃γ₂L and α₁β₂γ₂L GABAₐRs. The data show potentiation (mean ± SD; n = 5-6 oocytes) of current responses to low GABA by 50 μM DM490, DM492 and DM495, and 100 μM DM497 and DM489. No statistically significant difference between the two subtypes was observed.

Figure 6. Inhibitory activity of PAM-4 on the α₁β₂γ₂L GABAₐR. (A) Representative GABA (1 mM)-induced GABAₐR currents in the absence and presence of PAM-4. The modulator was coapplied with GABA during the desensitization decay phase of the response to GABA. The traces
are from different cells. (B) Representative currents induced by 10 µM GABA + 200 µM PAM-4 in the absence and presence of DM497. All traces are from the same cell. The dotted line in the first trace gives the level of peak response to 10 µM GABA in the absence of modulators in this cell. (C) The concentration-response relationships show the inhibitory effect of PAM-4 on currents elicited by 1 mM GABA, and the potentiating effect of DM497 on receptors activated by 10 µM GABA in the presence of 200 µM PAM-4. The dashed line gives the potentiating effect of DM497 alone (reproduced from Fig. 4D). The fitted IC₅₀ and EC₅₀ values are given in the text.

Figure 7. Comparison of inhibition and potentiation in α1β2γ2L and α1(V256S)β2γ2L GABA₅Rs. (A) Representative GABA₅R currents elicited by high (1 mM) GABA in the absence and presence of 50 µM DM490 or 100 µM PAM-4. The inhibitory effects of DM490 and PAM-4 are abolished in the mutant receptor. (B) Summary of findings. The α1(V256S) mutation abolishes inhibition by all tested compounds (wild-type: black circles, mutant: blue squares). (C) Representative GABA₅R currents elicited by low (1 µM in wild-type, 0.05 µM in the mutant) in the absence and presence of 50 µM DM490 or 200 µM PAM-4. The potentiating effect of DM490 is retained in the mutant receptor. The mutation also reveals a small potentiating effect of PAM-4 (bottom right trace). (D) Summary of findings. The α1(V256S) mutation does not modify potentiation by the DM compounds, but converts the effect of PAM-4 from inhibition to potentiation (P < 0.01 vs. no effect; paired t-test; Excel, Microsoft, Redmond, WA). The data for the wild-type receptor are reproduced from Figure 5.

Figure 8. Molecular docking of DM490, DM497, and PAM-4 in the α1β2γ2L GABA₅R model. (A) Stable docking sites for DM490 (red) at the β+/α− and α+/β− interfaces (left panel), DM497 (yellow) at the γ+/β− and α+/γ− interfaces (middle panel), and PAM-4 (blue) in the ion channel (right panel). Subunits (α, white; β, light-blue; and γ, red) are represented as ribbons, while ligands are represented as molecular surfaces. (B) RMSD plots for the 100-ns MD simulations for DM490,
DM497, and PAM-4 at each intersubunit site and at the ion channel site. \((\alpha^+\beta^+/\alpha^-)\) (blue dotted line), \((\gamma^+\beta^+/\alpha^-)\) (red dashed line), \((\alpha^+/\beta^-)\) (black dotted line), \((\gamma^+/\beta^-)\) (olive solid line), \((\alpha^+/\gamma^-)\) (black solid line), and ion channel lumen (blue solid line).

**Figure 9. Molecular interactions of DM490, DM497, and PAM-4 in the \(\alpha_1\beta_2\gamma_2L\) GABA\(_A\)R model.** DM490 at the \(\beta^+/\alpha^-\) (A) and \(\alpha^+/\beta^-\) (B) sites, and DM497 at the \(\gamma^+/\beta^-\) (C) and \(\alpha^+/\gamma^-\) (D) sites. (E) Molecular interactions for PAM-4 at the ion channel site. The ligands are represented as thin sticks, colored by atoms, with carbon atoms in green, while the interacting residues are represented by thick sticks, colored by atoms, where H atoms are omitted for clarity. Red dotted lines represent \(\pi-\pi\) interactions. The involved residues at each site are summarized in Table 5.

**Figure 10. Effects of allosteric agonists and mutations on potentiation by DM490.** (A-B) Comparison of DM490-induced potentiation of \(\alpha_1\beta_2\gamma_2L\) receptors activated by low concentrations of etomidate (Eto, 7-10 \(\mu\)M), phenobarbital (PhB, 0.75-1 mM), or GABA (0.5-1 \(\mu\)M). DM490 was applied at 50 \(\mu\)M. The current trace and summary data for GABA are reproduced from Figures 5 and 7. Statistical significance between the effects of DM490 on receptors activated by GABA, etomidate, or phenobarbital was determined using using one-way ANOVA (\(F(2,16) = 54.65, P < 0.001\)) followed by Dunnett's post-hoc multiple comparisons test (Stata/IC 12.1, StataCorp, College Station, TX, USA). * indicates significance at \(P < 0.01\). (C) Effects of mutations on receptor potentiation or activation by 50 \(\mu\)M DM490. The \(\alpha_1\beta_2(F289A)\gamma_2L\) and \(\alpha_1(Y293C)\beta_2\gamma_2L\) receptors were activated by low concentrations of GABA (0.1-0.2 \(\mu\)M) in the absence and presence of 50 \(\mu\)M DM490. The \(\alpha_1\beta_2\gamma_2L(L246W)\) and \(\alpha_1\beta_2\gamma_2L(F304C)\) receptors were constitutively active, shown by upward deflection of current upon the application of 200 \(\mu\)M picrotoxin (PTX). The effect of DM490 on the holding current in \(\alpha_1\beta_2\gamma_2L(L246W)\) and \(\alpha_1\beta_2\gamma_2L(F304C)\) receptors was measured by comparing the direct activating response to DM490, the inhibitory effect of picrotoxin, and the activating effect of 1 mM GABA + 50 \(\mu\)M propofol in the same cell. (D) The column graph summarizes DM490
effects on the α1β2(F289A)γ2L (β+/α− interface with the mutation in the β subunit), α1(Y293C)β2γ2L (α+/β− and α+/γ− interfaces with the mutation in the α subunit), α1β2γ2L(L246W) (α+γ− interface with the mutation in the γ subunit), and α1β2γ2L(F304C) (γ+/β− interface with the mutation in the γ subunit) receptors. The thick and thin dotted lines indicate mean ± 1 S.D. potentiation in the α1β2γ2L receptor. Statistical significance between the effects of DM490 on α1β2γ2L and the mutant receptors was determined by one-way ANOVA (F(4,21) = 83.00, P < 0.001) followed by Dunnett's post-hoc multiple comparisons test (*, P < 0.01; ns, P > 0.05).

Figure 11. Effects of an allosteric inhibitor and mutations on inhibition by PAM-4. (A) Representative GABA_A current elicited by 1 mM GABA in the absence and presence of 10 µM PS and PS + 200 µM PAM-4 in the α1β2γ2L receptor. Exposure to PS reduces steady-state current. Coapplication of PAM-4 enhances PS-induced inhibition. The inset shows the effects of PS and PAM-4 at higher ordinate resolution. (B) Comparison of the effects of 200 µM PAM-4 and 10 µM PS on current elicited by 1 mM GABA in the α1β2γ2L(L274C) receptor. The receptor is constitutively active as shown by apparent outward current upon exposure to 200 µM picrotoxin (PTX). (C) Comparison of the effects of 200 µM PAM-4 and 10 µM PS on current elicited by 1 mM GABA in the α1β2γ2L(T278C) receptor. Picrotoxin had minimal effect on holding current indicating low constitutive activity. (D) Summary of the effects of 200 µM PAM-4 and 10 µM PS on the α1β2γ2L(L274C), α1β2γ2L(T278C), and α1β2γ2L receptors. Statistical significance between the effects of PAM-4 on the wild-type and mutant receptors was determined using one-way ANOVA (F(2,12) = 35.55, P < 0.001) followed by Dunnett's post-hoc multiple comparisons test (Stata/IC 12.1, StataCorp, College Station, TX, USA). * indicates significance at P < 0.01.
Table 1. Molecular descriptors for the DM compounds, PAM-4 and PAM-2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MolVol (Å³)</th>
<th>H donor/acceptor</th>
<th>LogP</th>
<th>LogBB</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM497</td>
<td>193</td>
<td>1/1</td>
<td>3.52</td>
<td>0.39</td>
</tr>
<tr>
<td>DM490</td>
<td>199</td>
<td>0/1</td>
<td>3.17</td>
<td>0.30</td>
</tr>
<tr>
<td>DM480</td>
<td>200</td>
<td>1/2</td>
<td>2.78</td>
<td>-0.06</td>
</tr>
<tr>
<td>DM492</td>
<td>204</td>
<td>0/1</td>
<td>3.21</td>
<td>0.31</td>
</tr>
<tr>
<td>DM495</td>
<td>212</td>
<td>0/1</td>
<td>3.22</td>
<td>0.32</td>
</tr>
<tr>
<td>DM489 a</td>
<td>185</td>
<td>0/1</td>
<td>2.76</td>
<td>0.17</td>
</tr>
<tr>
<td>PAM-4 a</td>
<td>168</td>
<td>1/1</td>
<td>2.48</td>
<td>-0.06</td>
</tr>
<tr>
<td>PAM-2 a</td>
<td>182</td>
<td>1/1</td>
<td>2.96</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The table gives the molecular volume (MolVol), hydrogen-bonding capability (H donor/acceptor), and measures of lipophilicity (LogP) and ability to penetrate the blood-brain barrier (LogBB) for the tested DM compounds. All parameters were calculated using the Biovia Discovery Studio software (Dassault Systèmes Co., MA, USA). High LogP indicates high lipophilicity. LogBB values between 0 and 0.7 and between -0.01 and -0.52 indicate high and medium penetration, respectively. a The LogP and LogBB values for DM489 and PAM-2 (Arias et al., 2020) and PAM-4 (Bagdas et al., 2021) are included for reference.
Table 2. Pharmacological effects of the novel DM compounds on apparent affinity to GABA in the α1β3γ2L GABA\_3R.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_d,_{GABA}$ (number of cells), μM</th>
<th>$K_d,_{GABA \ (DM)}$ (number of cells), μM</th>
<th>$K_d,<em>{GABA} / K_d,</em>{GABA \ (DM)}$ (fold difference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM497</td>
<td>16.8 ± 1.6 (22)</td>
<td>0.2 ± 0.2 (16)</td>
<td>84</td>
</tr>
<tr>
<td>DM490</td>
<td>16.5 ± 1.2 (15)</td>
<td>0.2 ± 0.02 (14)</td>
<td>75</td>
</tr>
<tr>
<td>DM492</td>
<td>16.5 ± 1.2 (15)</td>
<td>0.3 ± 0.03 (18)</td>
<td>63</td>
</tr>
<tr>
<td>DM489</td>
<td>19.0 ± 1.1 (16)</td>
<td>2.1 ± 0.2 (12)</td>
<td>9.1</td>
</tr>
<tr>
<td>DM495</td>
<td>13.0 ± 1.1 (20)</td>
<td>1.5 ± 0.1 (15)</td>
<td>8.4</td>
</tr>
<tr>
<td>DM480</td>
<td>13.0 ± 1.1 (20)</td>
<td>4.6 ± 0.4 (16)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

$K_d,_{GABA}$ and $K_d,_{GABA \ (DM)}$ reflect the apparent binding affinities to GABA in the absence or presence of 100 μM of each tested DM compound. The table gives means ± propagated errors (numbers of cells) for $K_d,_{GABA}$ and $K_d,_{GABA \ (DM)}$ calculated using Eq. (2).
Table 3. GABAergic properties of the novel DM compounds and PAM-4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC\textsubscript{50} or IC\textsubscript{50}, µM</th>
<th>n\textsubscript{H}</th>
<th>Apparent efficacy, % of control (n cells)</th>
<th>K\textsubscript{R}, µM</th>
<th>c</th>
<th>∆G, kcal/mol</th>
<th>Inhibition, % of control (n cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM490</td>
<td>12 [10 to 15] \textsuperscript{a}</td>
<td>1.3 ± 0.2</td>
<td>519 ± 260 \textsuperscript{c} (5)</td>
<td>22 ± 3</td>
<td>0.22 ± 0.05</td>
<td>-1.79 ± 0.28</td>
<td>64 ± 20 (5)</td>
</tr>
<tr>
<td>DM497</td>
<td>32 [20 to 50] \textsuperscript{a}</td>
<td>1.5 ± 0.3</td>
<td>540 ± 523 \textsuperscript{c} (5)</td>
<td>50 ± 33</td>
<td>0.39 ± 0.16</td>
<td>-1.23 ± 0.67</td>
<td>60 ± 15 (5)</td>
</tr>
<tr>
<td>DM492</td>
<td>15 [11 to 19] \textsuperscript{a}</td>
<td>2.1 ± 0.7</td>
<td>398 ± 165 \textsuperscript{c} (6)</td>
<td>19 ± 7</td>
<td>0.41 ± 0.16</td>
<td>-1.13 ± 0.45</td>
<td>55 ± 12 (5)</td>
</tr>
<tr>
<td>DM489</td>
<td>ND</td>
<td>ND</td>
<td>144 ± 9 \textsuperscript{d} (5)</td>
<td>ND</td>
<td>0.83 ± 0.03</td>
<td>-0.23 ± 0.04</td>
<td>67 ± 7 (5)</td>
</tr>
<tr>
<td>DM495</td>
<td>ND</td>
<td>ND</td>
<td>189 ± 53 \textsuperscript{d} (5)</td>
<td>ND</td>
<td>0.79 ± 0.14</td>
<td>-0.30 ± 0.21</td>
<td>54 ± 15 (5)</td>
</tr>
<tr>
<td>PAM-4</td>
<td>54 ± 7 \textsuperscript{b}</td>
<td>-6.4 ± 9.2</td>
<td>50 ± 4 \textsuperscript{c} (5-11)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>81 ± 7 (6)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Experiments were performed at low [GABA] (<EC\textsubscript{5,15}; PA<0.2) (Fig. 3). EC\textsubscript{50}s are given as geometric means [95% confidence intervals].

\textsuperscript{b} The IC\textsubscript{50} for PAM-4 was estimated from pooled data using the Hill equation. The numbers of cells were: 5 at 1 µM, 7 at 10 µM, 5 at 50 µM, 5 at 100 µM, 11 at 200 µM, and 7 at 500 µM PAM-4. The IC\textsubscript{50} is given as best-fit parameter ± standard error of the fit.

\textsuperscript{c} Apparent efficacy is calculated as the ratio of Hill-fitted Y\textsubscript{max} to the peak response to GABA alone, expressed in % of control (100% = no effect).

\textsuperscript{d} Estimated at 50 µM concentration of the compound.

\textsuperscript{e} c is a measure of efficacy, estimated from fitting a potentiation concentration-response relationship to Eq. (3). For DM489 and DM495, the value of c was calculated from \( P_{A,50} \mu M = \frac{1}{1 + L \times c_{50} \mu M} \).

\textsuperscript{f} ∆G is a measure of efficacy, estimated from fitting a potentiation concentration-response relationship to Eq. (3). For DM489 and DM495, the value of ∆G was calculated from \( \frac{1}{1 + L \times c_{50} \mu M} \).
ÅG was calculated as NRT\times \ln(c), with the number of binding sites (N) constrained to 2.

Inhibition was measured during a steady-state response to 1 mM GABA, and shows the response remaining following exposure to GABA + 50 µM modulator.

ND, not determined.
Table 4. Molecular docking and stability of DM490, DM497, and PAM-4 at different subunit interfaces and the ion channel of the α1β2γ2 GABAA-R model.

<table>
<thead>
<tr>
<th>Docking site</th>
<th>DM490</th>
<th></th>
<th>DM497</th>
<th></th>
<th>PAM-4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBE</td>
<td>RMSD (VAR)</td>
<td>TBE</td>
<td>RMSD (VAR)</td>
<td>TBE</td>
<td>RMSD (VAR)</td>
</tr>
<tr>
<td>β+/α− (anesthetic) *</td>
<td>-42</td>
<td>2.9 (0.1)</td>
<td>-43</td>
<td>7.1 (0.1)</td>
<td>-36</td>
<td>3.9 (0.2)</td>
</tr>
<tr>
<td>α+/β− (anesthetic)</td>
<td>-38</td>
<td>5.1 (0.1)</td>
<td>-38</td>
<td>11.3 (1.0)</td>
<td>-38</td>
<td>13.9 (1.0)</td>
</tr>
<tr>
<td>γ+/β− (anesthetic)</td>
<td>-35</td>
<td>5.7 (1.6)</td>
<td>-39</td>
<td>4.9 (0.1)</td>
<td>-32</td>
<td>12.4 (0.8)</td>
</tr>
<tr>
<td>α+/γ− (orphan)</td>
<td>-41</td>
<td>17.5 (16.3)</td>
<td>-40</td>
<td>8.0 (0.1)</td>
<td>-29</td>
<td>4.1 (0.9)</td>
</tr>
<tr>
<td>Ion channel</td>
<td>-40</td>
<td>3.7 (0.4)</td>
<td>-34</td>
<td>11.1 (2.9)</td>
<td>-27</td>
<td>5.2 (0.1)</td>
</tr>
</tbody>
</table>

TBE: Theoretical binding energy (kcal/mol). More negative values indicate higher binding affinity. Depending on the RMSD VAR values, measured during the last third of the MDs, the observed interactions were considered stable (RMSD VAR <0.2), nearly stable (RMSD VAR 0.2-2), and unstable (RMSD VAR >2).

*Stable interaction within the (γ+)β+/α− interface.
Table 5. Molecular interactions of DM490, DM497, and PAM-4 at the subunit interfaces and ion channel of the α1β2γ2 GABA<sub>Α</sub>R model.

<table>
<thead>
<tr>
<th>Docking Site</th>
<th>DM490</th>
<th>DM497</th>
<th>PAM-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intersubunit Sites</td>
<td>(+) side</td>
<td>(-) side</td>
<td>(+) side</td>
</tr>
<tr>
<td>γ/β (A)</td>
<td>Near stable</td>
<td>TM2: T277 S280 TM3: S301 F304 I305 F308</td>
<td>TM1: L223 Q224 M227 P228 L231</td>
</tr>
</tbody>
</table>

Ion Channel Site

| γ subunit | Not found | Not found | TM2: L274 (9’), T277, T278 (13’) |
| β subunit | Near stable | Unstable | TM1: I232 TM2: I255, T256, L259 (9’), T260, T263 (13’), I264 |
| α subunit | | | TM2: T261 (6’), T265 |

The "+" and "−" sides indicate contributions made by the two sides of an intersubunit interface within a counter-clockwise order of subunits.

Residues in bold: π-π interactions. A: Anesthetic site; O: Orphan site.
Residues at 6’, 9’, and 13’ correspond to the canonical TM2 rings in the ion channel lumen.
Figure 1

A

\[
\begin{align*}
\text{DM497} & \quad \text{(R=O,S)} \\
\text{DM492} & \\
\text{DM495} & \\
\text{DM480} & \\
\text{PAM-4} & \\
\text{PAM-2} & \quad \text{(DM490)}
\end{align*}
\]
Figure 2

![Image of cellular imaging data showing effects of different GABA concentrations and treatments with DM497, DM490, DM492, DM495, DM489, and DM480.](molpharm.aspetjournals.org)
Figure 3

A-B: Graphs showing the effect of different concentrations of DM497 and DM490 on F/F₀ against Log[GABA].

C-D: Graphs showing the effect of different concentrations of DM492 and DM495 on F/F₀ against Log[GABA].

E-F: Graphs showing the effect of different concentrations of DM489 and DM480 on F/F₀ against Log[GABA].

G-H: Graphs showing the effect of different concentrations of DM497 and DM490 on F/F₀ against concentration.
Figure 4

A

GABA + 1 μM DM490 + 10 μM DM490 + 100 μM DM490

20 s 0.10 μA

B

GABA + 1 μM DM497 + 10 μM DM497 + 100 μM DM497

30 s 0.20 μA

C

GABA + 1 μM DM492 + 10 μM DM492 + 100 μM DM492

30 s 0.20 μA

D

![Graph showing data for DM490, DM497, and DM492](molpharm.aspetjournals.org)

P_A

[ Ligand ] (μM)

1 10 100
Figure 5

The graph shows the multiples of control for different samples labeled DM490, DM497, DM492, DM489, and DM495. The data is represented by various symbols indicating two subtypes: α1β3γ2L and α1β2γ2L.

- **DM490**: The data points for α1β3γ2L are higher than those for α1β2γ2L, indicating a higher multiple of control for the former subtype.
- **DM497**: The data points are relatively close, with a slight advantage for α1β3γ2L.
- **DM492**: Similar to DM497, the data points are close, with a slight advantage for α1β3γ2L.
- **DM489**: The data points are lower compared to the other samples, with a noticeable advantage for α1β2γ2L.
- **DM495**: The data points are consistent with the other samples, showing a slight advantage for α1β3γ2L.

Overall, the graph illustrates the comparative multiples of control for different samples and subtypes, with α1β3γ2L generally showing a higher multiple of control compared to α1β2γ2L.
Figure 6

A

10 µM PAM-4

GABA

100 s 0.2 µA

50 µM PAM-4

GABA

0.15 µA

100 µM PAM-4

GABA

0.1 µA

200 µM PAM-4

GABA

0.1 µA

B

GABA + 200 µM PAM-4

20 s 0.4 µA

+1 µM DM497

+10 µM DM497

+100 µM DM497

C

P_A or normalized response

[1.0, 0.8, 0.6, 0.4, 0.2, 0.0]

[Ligand] (µM)

PAM-4

200 µM PAM-4 + DM497

DM497

---
Figure 11

A. α1β2γ2L

GABA  PS  PAM-4

40 s  1 μA  200 nA

B. α1β2γ2L(L274C)

GABA  PAM-4  PS  PTX

100 s  200 nA

C. α1β2γ2L(T278C)

GABA  PAM-4  PS  PTX

100 s  100 nA

D. α1β2γ2L(L274C)  α1β2γ2L(T278C)  α1β2γ2L

% of control

<table>
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<th>PAM-4</th>
<th>PS</th>
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<tr>
<td>α1β2γ2L(L274C)</td>
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<tr>
<td>α1β2γ2L(T278C)</td>
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</tbody>
</table>

* indicates statistically significant differences.