Supporting information

Structure-Activity studies of 3,9-Diazaspiro[5.5]undecane-based γ-Aminobutyric Acid Type A Receptor Antagonists with Immunomodulatory Effect

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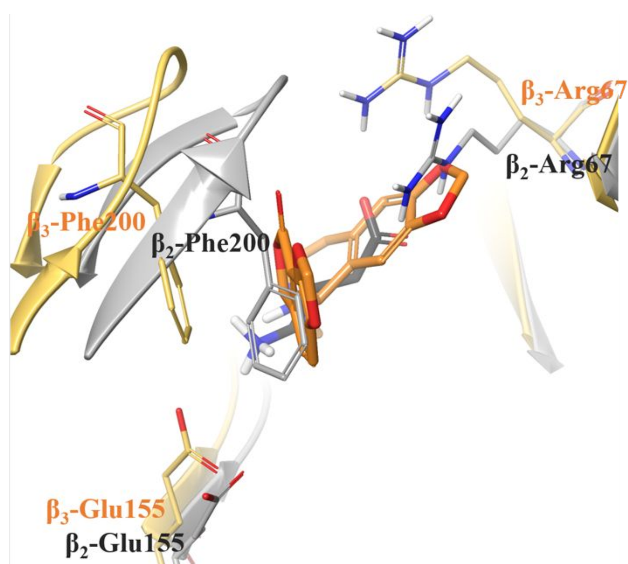


Figure S1. Comparison between the degree of opening of the binding site, depending on the complexed ligand. The loop C (represented by Phe200) of the β_2/α_1 interface (grey backbone and residues) complexed with the agonist GABA (grey ligand, PDB ID: 6D6T) is tightly closed on the ligand. Instead, the loop C of the β_3/α_1 interface (orange backbone and residues) complexed with the antagonist bicuculine (orange ligand, PDB ID: 6HUK) is pushed outward by the bulky ligand.

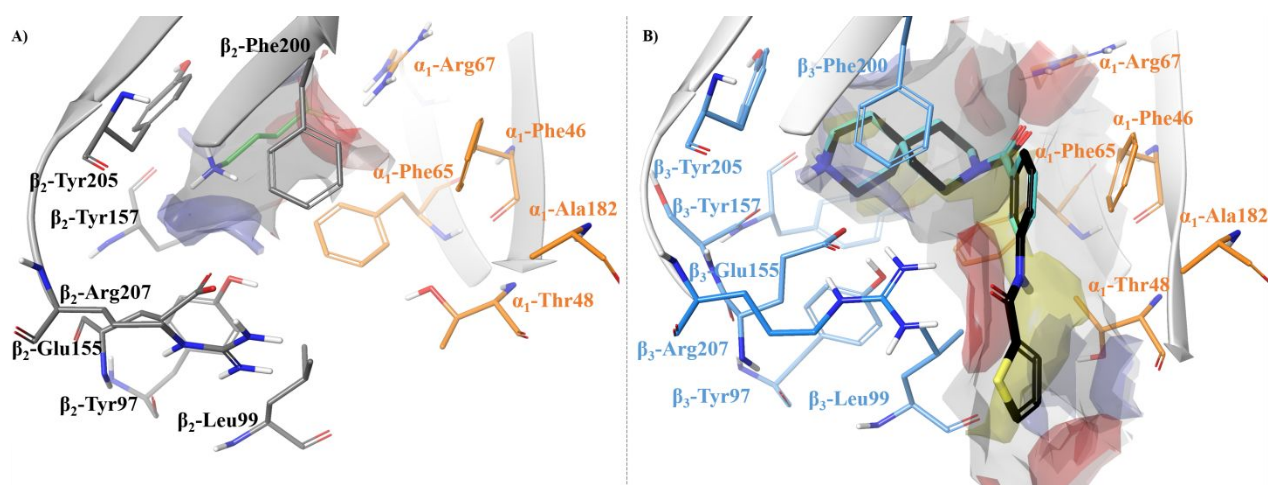


Figure S2. Binding site map calculated with SiteMap of A) the β_2/α_1 binding site (PDB ID: 6D6T) complexed with GABA (green) compared to B) the β_3/α_1 binding site upon docking of **018** (black) and **1d** (cyan) (obtained from PDB ID: 6HUK). The inner surface of the receptors are depicted in grey, while the areas where the presence of hydrogen-bond acceptors, hydrogen-bond donors and

lipophilic moieties are favored are displayed respectively in red, blue and yellow. The subpocket is not accessible in GABA-bound 3D models.

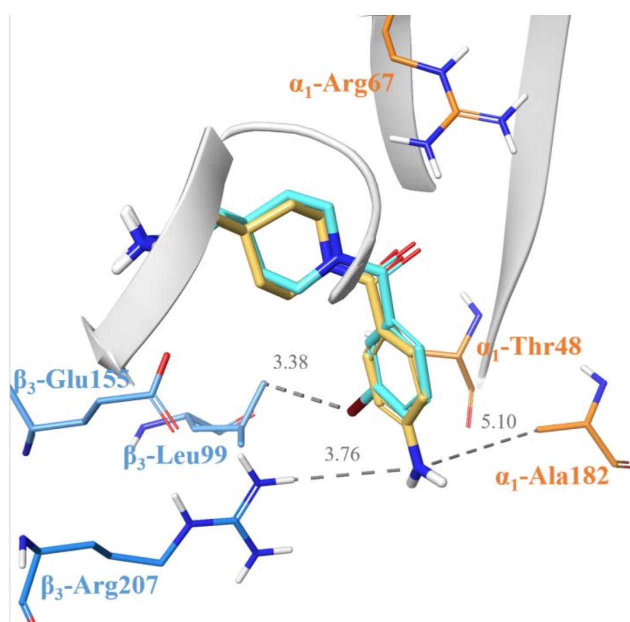


Figure S3. Docking poses of **1h** (cyan) and of **1p** (gold) at the β_3/α_1 binding site (PDB ID: 6HUK). Distances between the *m*- or *p*- substituent and respectively Leu99 and Arg207/Ala182 are shown in grey.

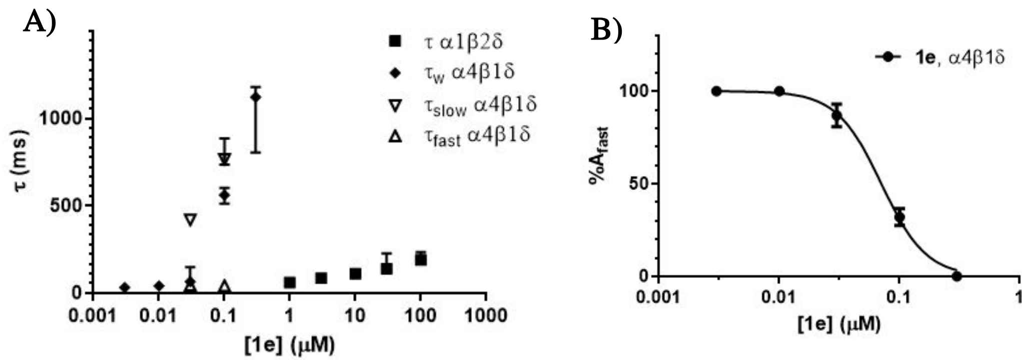


Figure S4. A) Activation time constants, τ, for currents induced by GABA ($EC_{90\text{-}100}$) with pre-application of varying concentrations of **1e** on α1β2δ and α4β1δ receptors, measured by whole-cell patch-clamp recording. τ values were determined by monoexponential curve fitting except for 0.03 μM and 0.1 μM **1e** on α4β1δ receptors where a slow and fast phase of receptor activation could be resolved by biexponential curve fitting resulting in $τ_{fast}$ and $τ_{slow}$, respectively (open symbols). For the α4β1δ receptor, a weighted τ value ($τ_w$) is shown. This is a weighted average of the $τ_{fast}$ and $τ_{slow}$ values, weighted by their fractional contribution to the total current amplitude. For the concentrations where monoexponential fitting was used, $τ_w$ is just the single τ values obtained. Data are shown as median ± interquartile range for 4–12 cells.

For the α4β1δ receptor, the $τ_{fast}$ (0.03 μM and 0.1 μM) is similar to τ-values for the lowest concentrations of **1e** (0.003 μM and 0.01 μM) and to τ for GABA applied alone (43 ms [35; 52]), confirming that the fast component of activation is indeed due to GABA activation of vacant receptors. The τ obtained with 0.3 μM **1e** on α4β1δ receptors (where $\%A_{fast} = 0$) represents the situation where all receptors are occupied by **1e** at the time where GABA is applied, and thus this τ value (1.12 s [0.81; 1.18]) reflects the dissociation rate of **1e** from the receptors. The $τ_{slow}$ for 0.1 μM **1e** is not significantly different, but the $τ_{slow}$ for 0.03 μM **1e** is significantly smaller than the τ for 0.3 μM **1e** ($p<0.001$, Kruskal-Wallis ANOVA followed by Dunn's multiple comparison). This trend towards faster $τ_{slow}$ has been observed previously with **018**[26] and can be explained by the fact that at the lower concentrations of antagonist, the fractional amplitude of the slow component ($\%A_{slow}$) is small, thus giving less reliable estimates of the $τ_{slow}$.

For the α1β2δ receptor, it was not possible the resolve fast and slow components of receptor activation at any concentration. This is likely due to the τ value for dissociation of **1e** from the α1β2δ receptor being faster and therefore closer to the $τ_{fast}$ for GABA activation of the vacant receptor. The monoexponential τ values obtained for **1e** at the α1β2δ receptor are thus hybrids of the underlying

τ_{fast} and τ_{slow} where the contribution of τ_{fast} decreases with **1e** concentration and the (hybrid) τ value increases accordingly (similar to what is seen with τ_w at α4β1δ receptors). It is apparent from the figure that, at α1β2δ receptors, considerably higher concentrations of **1e** are required to associate to the receptor and increase the τ value over the value from GABA activation of the vacant receptor Also, the τ obtained with highest concentration of **1e** (189 ms [176; 235]) is approximately 6-fold faster than for the α4β1δ receptor.

B) The fractional contribution of the fast components of receptor activation to the total current amplitude ($\%A_{fast}$) decreases as a function of the concentration of **1e** on α4β1δ receptors. $\%A_{fast}$ represents the fraction of receptors not occupied by **1e** at the end of the 20 s preapplication, just before GABA is applied. Data are shown as mean ± SEM or 4–12 cells. The concentration of **1e** corresponding to 50 $\%A_{fast}$ was estimated by curve fitting to 71 nM (pIC_{50} ±SEM = 7.152 ± 0.029).

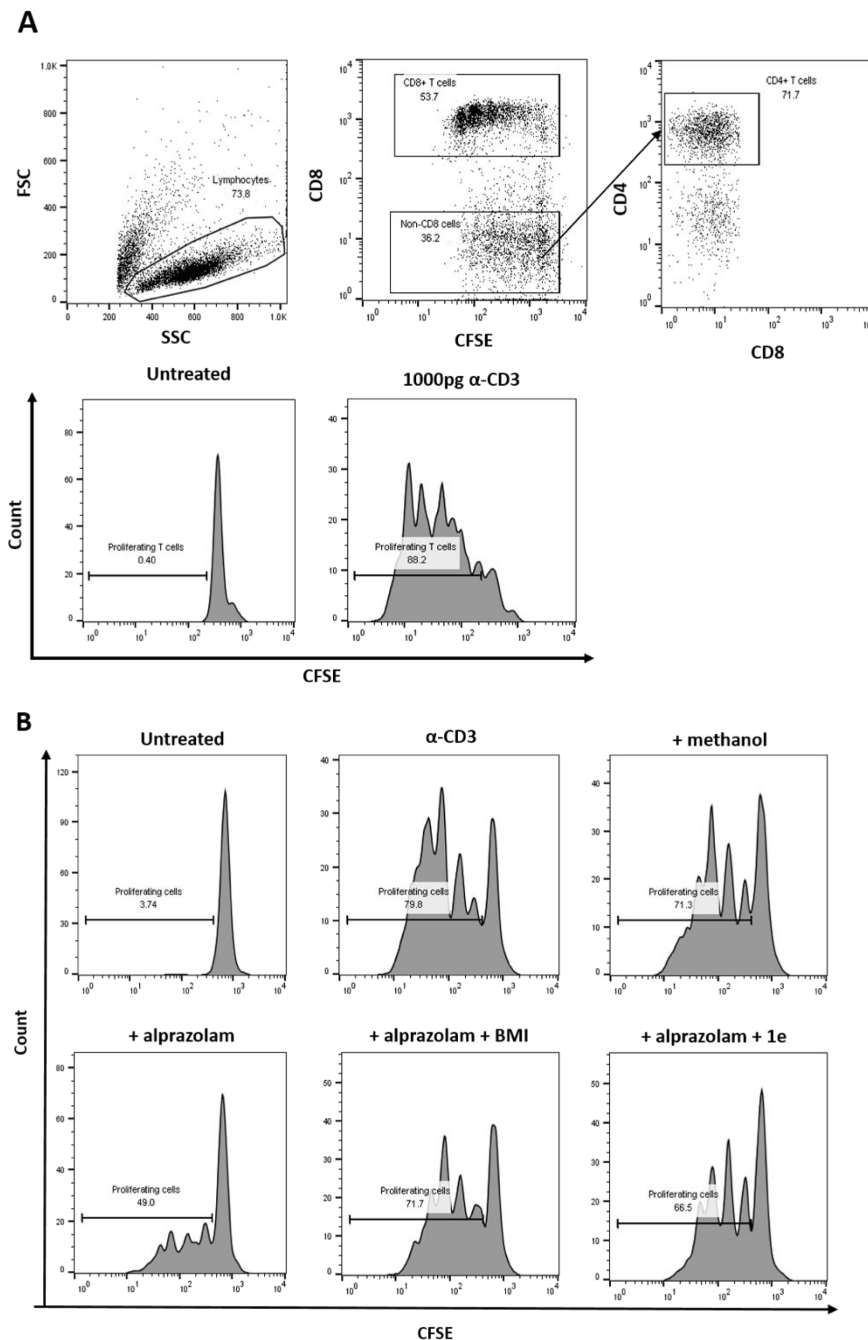
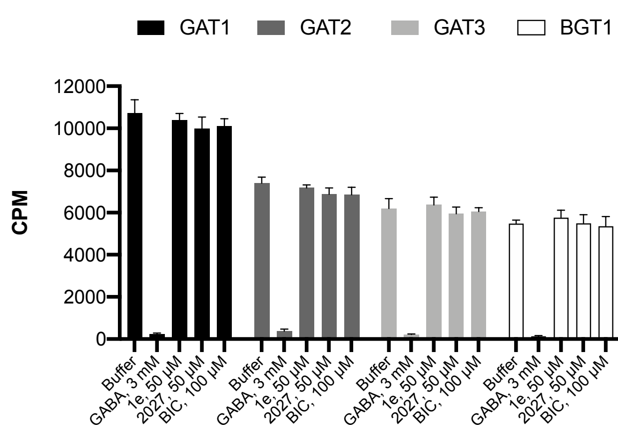


Figure S5. A) Representative flow cytometry plots depicting the gating strategy used to identify cell populations of interest from PBMC and splenocyte preparations. Lymphocytes were gated according to size (forward scatter; FSC) and granularity (side scatter; SSC). Within the lymphocyte gate, CD8+ and CD4+ T cells were further gated on using established lineage markers for these cells (CD8 and CD4). Histograms were used to determine the percentage of proliferating cells present under each condition, compared to an untreated control. Stimulation with α-CD3 alone was used as a positive control of proliferation. B) shows exemplar flow cytometry plots (from human CD8+ T cells)

depicting the recovery of inhibition of proliferation induced by alprazolam in response to treatment with both BMI and **1e**.

A)

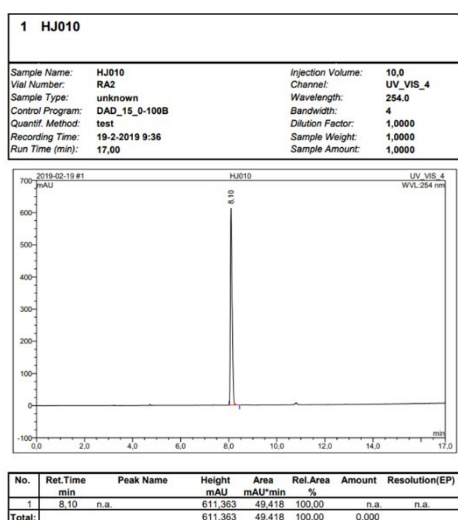


B)

	nAChR α7	nAChR α4β2	5-HT_{1B}	5-HT_{2B}	5-HT_3	5-HT_7	GABA-T
% Inh (**1e**, 50 µM, n=2)	-1	62	16	42	68	-12	0

Figure S6. A) Lack of activity of **1e** at the four human GABA transporters tested in the [^{3}H]GABA uptake assay. Data presented are means ± S.D. of triplicate measurements of a single representative experiment. One additional independent experiment gave similar results. The [^{3}H]GABA competition uptake assay in CHO cells stably expressing each of the four human GABA transporters was performed as previously described.[1] Briefly, on day 1, cells were seeded in white 96-well plates. On day 2, assay was performed as follows: Medium was removed and cells were washed with 100 µl/well assay buffer (HBSS supplemented with 20 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH=7.4). Then, 75 µl/well assay buffer containing 30 nM [^{3}H]GABA and the test compounds (50 µM of 1e/2027, 100 µM bicuculline; bic, and GABA 3 mM as positive control) were added to the cells followed by incubation for 3 min at 37 ˚C. The cells were washed 3 times with 100 µl/well ice-cold assay buffer, 150 µl/well MicroScint™20 (PerkinElmer, Boston, MA, USA) added, plated sealed followed by shaking for 1 hour. The plate was counted in a Packard TopCounter microplate scintillation counter (PerkinElmer). Data are depicted as counts per minute (CPM) compared to the

GABA control. B) Lack of activity or low binding of **1e** in competition binding assays at a panel of human receptors. No activity of **1e** at rat GABA-T, measured in a spectrofluorimetric enzymatic assay. Data are represented as means of duplicate measurements of a single representative experiment. Radioligand binding assays at α7 nAChR ([^{3}H] Methyllycaconitine), α4β2 nAChR ([^{3}H] Cytisine), 5-HT_{1B} ([^{3}H] GR125743), 5-HT_{2B} (^{3}[H] Mesulergine), 5-HT3 ([^{3}GR-65630]), 5-HT_7 ([^{3}H] Lysergic acid diethylamide) and spectrofluorimetric enzymatic assay at GABA-T (quantitation of succinic) were performed by Eurofins Panlabs Discovery Services Taiwan, Ltd.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	8,10	n.a.	611,363	49,418	100,00	n.a.	n.a.
Total:			611,363	49,418	100,00	0,000	

Figure S7. HPLC trace of **1e** (254 nm).

REFERENCES

1. Al-Khawaja, A.; Petersen, J. G.; Damgaard, M.; Jensen, M. H.; Vogensen, S. B.; Lie, M. E. K.; Kragholm, B.; Bräuner-Osborne, H.; Clausen, R. P.; Frølund, B.; Wellendorph, P. Pharmacological Identification of a Guanidine-Containing β-Alanine Analogue with Low Micromolar Potency and Selectivity for the Betaine/GABA Transporter 1 (BGT1). *Neurochemical Research* **2014**, *39*, 1988-1996.