

Supplemental Data MOLPHARM-AR-2020-000067

A benzodiazepine ligand with improved GABA_A receptor α 5-subunit-selectivity driven by interactions with loop C

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Supplemental Figure 1: Nomenclature of mutated subunits and sequence alignment

Supplemental Figure 1 shows the sequences of rat α 3 and α 5 subunits, as published by UniProtKB (accession IDs P20236 and P19969). The point mutated amino acids are highlighted in yellow. In the structural work, amino acids of the mature protein are counted without the signal peptide, while in the mutational analysis, amino acids are counted including the signal peptide. In addition a full alignment of human α 1, α 3 and α 5 subunits is shown to support Figure 3 in the manuscript.

GBRA3_RAT (P20236) signal peptide (SP): 28 amino acids

10	20	30	40	50	
MITTQMWHFY	VTRVGLLLLI	SILPGTTGQG	ESRRQEPGDF	VKQDIGGLSP	
60	70	80	90	100	
KHAPDIPDDS	TDNITIFTRI	LDRLLDGYDN	RLRPLGDAV	TEVKTDIYVT	
110	120	130	140	150	
SFGPVSDDTM	EYTIDVFFRQ	TWHDRLKFD	GPMKILPLNN	LLASKIWTPD	
160	170	180	190	200	
TFFHNGKKS	AHNMTTPNKL	LRLVDNGTLL	YTMRLTIHAE	CPMHLEDFPM	T215 (with Sp)
210	220	230	240	250	T187 (without Sp)
DVHACPLKFG	SYAYTKAEVI	YSWTLGKNKS	VEVAQDGSRL	NQYDLLGHVV	
260	270	280	290	300	S257 (with Sp)
GTEIIRSGSTG	EYVVMTHFH	LKRKIGYFVI	QTYLPCIMTV	ILSQVSFWLN	S229 (without Sp)
310	320	330	340	350	
RESVPARTVF	GVTTVLMTT	LSISARNSLP	KVAYATAMDW	FMAVCYAFVF	
360	370	380	390	400	
SALIEFATVN	YFTKRSWAVE	GKKVPEALEM	KKKTPAAPTK	KTSTTFNIVG	
410	420	430	440	450	
TTYPINLALD	TEFSTISKAA	AAPSASSTPT	VIASPKTTYV	QDSPAETKTY	
460	470	480	490		
NSVSKVDKIS	RIIFPVLF	FNLVYWATYV	NRESAIKGM	RKQ	

GBRA5_RAT (P19969) Signal peptide: 25 amino acids

10	20	30	40	50	
MDNGMLSRFI	MTKTLVFCI	SMTLSHF	SQMPSTSSVQD	ETNDNITIFT	
60	70	80	90	100	
RILDGLLDGY	DNRLRPGLGE	RITQVRTDIY	VTSFGPVSDDT	EMEYTTIDVFF	
110	120	130	140	150	
RQSWKDERLR	FKGPMQRLPL	NNLLASKIWT	PDTFFHNGKK	SIAHNMTTPN	P197 (with Sp)
160	170	180	190	200	P172 (without Sp)
KLLRLEDDGT	LLYTMRLTIS	AECPMQLEDF	PMDAHACPLK	FGSYAYP	
210	220	230	240	250	T239 (with Sp)
VVYVWTNGST	KSVVVAEDGS	RLNQYHLMGQ	TVGTENIS	TGEYTIMTAH	T214 (without Sp)
260	270	280	290	300	
FHLKRKIGYF	VIQTYLPCIM	TVILSQVSFW	LNRESVPART	VFGVTTVLTM	
310	320	330	340	350	
TTLISISARNS	LPKVAYATAM	DWFIACVYAF	VFSALIEFAT	VNYFTKRGWA	
360	370	380	390	400	
WDGKKALEEA	KIKKKERELI	LNKSTNAFTT	GKLTHPPNIP	KEQLPGGTGN	
410	420	430	440	450	
AVGTASIRAS	EEKTSESKKT	YNSISKIDKM	SRIVFPILFG	TFNLVYWATY	
460					
LNREPVIKGA	TSPK				

Full alignment of human $\alpha 1$, $\alpha 3$ and $\alpha 5$ subunits

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hgABRa1 -----QFSLDRLKDNITVPRILDRLLDGYDNRRLPGLGRRVTVKTDIFVTSFGPVSDHDMETIIVVFRQSWKDRLLFKGEMTVLRRLNMLMASKIWPDPDTPFRNGKKSVAHNMTPFNKLLRLEDDGT
a1Rat_28-455 -----QFSLDRLKDNITVPRILDRLLDGYDNRRLPGLGRRVTVKTDIFVTSFGPVSDHDMETIIVVFRQSWKDRLLFKGEMTVLRRLNMLMASKIWPDPDTPFRNGKKSVAHNMTPFNKLLRLEDDGT
hgABRa5 -----SHFGFSQMPSTSSVQDEENNITIFTRILDGLLDGYDNRRLPGLGRRITQVRDIYVTSFGPVSDTMEYTIIVVFRQSWKDRLLFKGEMTVLRRLNMLLASKIWPDPDTPFRNGKKSIAHNMTPFNKLLRLEDDGT
a5Rat_26-464 -----SHFGFSQMPSTSSVQDEENNITIFTRILDGLLDGYDNRRLPGLGRRITQVRDIYVTSFGPVSDTMEYTIIVVFRQSWKDRLLFKGEMTVLRRLNMLLASKIWPDPDTPFRNGKKSIAHNMTPFNKLLRLEDDGT
hgABRa3 QGESRRRQPGDFVKQDIGGLSPKHADIPDSDTNDITIFTRILDRLLDGYDNRRLPGLGDVAVKTKDIYVTSFGPVSDTMEYTIIVVFRQSWHDERLKFDPGPKILPLNLLASKIWPDPDTPFRNGKKSVAHNMTPFNKLLRLEVINGT
a3Rat_29-493 QGESRRRQPGDFVKQDIGGLSPKHADIPDSDTNDITIFTRILDRLLDGYDNRRLPGLGDVAVKTKDIYVTSFGPVSDTMEYTIIVVFRQSWHDERLKFDPGPKILPLNLLASKIWPDPDTPFRNGKKSVAHNMTPFNKLLRLEVINGT
1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120.....130.....140.....150

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hgABRa1 LLYTMRLTVRAECPMHLLEDPPMDAHAQPLKFGSYAYTRAEVVVEWIREPARSVVVAEDGSRILNQYDLLGQTVDSGIVQSSSTGEYVVMTHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
a1Rat_28-455 LLYTMRLTVRAECPMHLLEDPPMDAHAQPLKFGSYAYTRAEVVVEWIREPARSVVVAEDGSRILNQYDLLGQTVDSGIVQSSSTGEYVVMTHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
hgABRa5 LLYTMRLTVRAECPMHLLEDPPMDAHAQPLKFGSYAYPNSEVVVVMNGSTKSVVVAEDGSRILNQYHLMGQTVGTENISTSTGEYIMTAHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
a5Rat_26-464 LLYTMRLTVRAECPMHLLEDPPMDAHAQPLKFGSYAYPNSEVVVVMNGSTKSVVVAEDGSRILNQYHLMGQTVGTENISTSTGEYIMTAHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
hgABRa3 LLYTMRLTVRAECPMHLLEDPPMDVHAQPLKFGSYAYTTAEVVVSWLGGKNSVEVAEDGSRILNQYDLLGHVVGTEIIRSESGEYVVMTHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
a3Rat_29-493 LLYTMRLTVRAECPMHLLEDPPMDVHAQPLKFGSYAYTTAEVVVSWLGGKNSVEVAEDGSRILNQYDLLGHVVGTEIIRSESGEYVVMTHPFLKRRIGYFVIQTYLPCIMTVILSQVSWFLNRESVPARTVPGVTVLIMTILSISARNS
.....160.....170.....180.....190.....200.....210.....220.....230.....240.....250.....260.....270.....280.....290.....300

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hgABRa1 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRGYAWDGR-SVVPKPKRVKDFLIK--KNTVAPTASVTPHLARGDPGLATIAKSE-----ATIEPKVKP-ETKPPSP--KKTFNSSVKRIDRLSRIAPPLLEPGIFHLVYWA
a1Rat_28-455 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRGYAWDGR-SVVPKPKRVKDFLIK--KNTVAPTASVTPHLARGDPGLATIAKSE-----ATIEPKVKP-ETKPPSP--KKTFNSSVKRIDRLSRIAPPLLEPGIFHLVYWA
hgABRa5 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRGWAWGKKALBAKIKKK-REVLNKSNAFTPKKSGHPPIPK-FOPPAGTS-----NMT-FVSVVPEKETSSE--KKTYSNSIKIDKMSRIVFPVLPQPHLVYWA
a5Rat_26-464 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRGWAWGKKALBAKIKKKREELINKSTNAPTQGLKHPPIPK-QLPQGGT-----NAVGTASIRASEKTSSE--KKTYSNSIKIDKMSRIVFPVLPQPHLVYWA
hgABRa3 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRSWAWGKKVPALEMKKKTTPAAPAKKTSSTFNIVGTTVPINLAK-ETEPSTISKGA-AAPASSTETIIASPKATVQDSPTETKTYNSVSKVKDISRIIPVLPALPHLVYWA
a3Rat_29-493 LPKVAYATAMDFPIAVCYAPVFSALIEPATVNYPTKRSWAWGKKVPALEMKKKTTPAAPAKKTSSTFNIVGTTVPINLAK-ETEPSTISKAAAAPASSTETVVIASPKATVQDSPTETKTYNSVSKVKDISRIIPVLPALPHLVYWA
.....310.....320.....330.....340.....350.....360.....370.....380.....390.....400.....410.....420.....430.....440.....450

**:*:
hgABRa1 TYLNREPOLKAPPHQ
a1Rat_28-455 TYLNREPO-----
hgABRa5 TYLNREPVIKGASPK
a5Rat_26-464 TYLNREPVIKGASPK
hgABRa3 TYVHRESAIGHIRKQ
a3Rat_29-493 TYVHRESAIGHIRKQ
.....460.....

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Supplemental Tables 1 and 2: Comparison of rat and human subunits

Throughout our manuscript, amino acids are counted with the signal peptide included and based on the rat sequences. In some published studies, human sequences are used and/or the signal peptide is not counted, and the first amino acid of the mature peptide numbered as “1”. In Table 1 the different length of the signaling peptides is shown, in Table 2 the amino acids analyzed in this study are compared between rat and human sequence.

Table 1: Length of signaling peptide

Length of the signaling peptide as published by UniProtKB (accession IDs P62813, P20236, P19969, P14867, P34903, and P31644)

	rat	human
$\alpha 1$	27	27
$\alpha 3$	28	28
$\alpha 5$	25	31

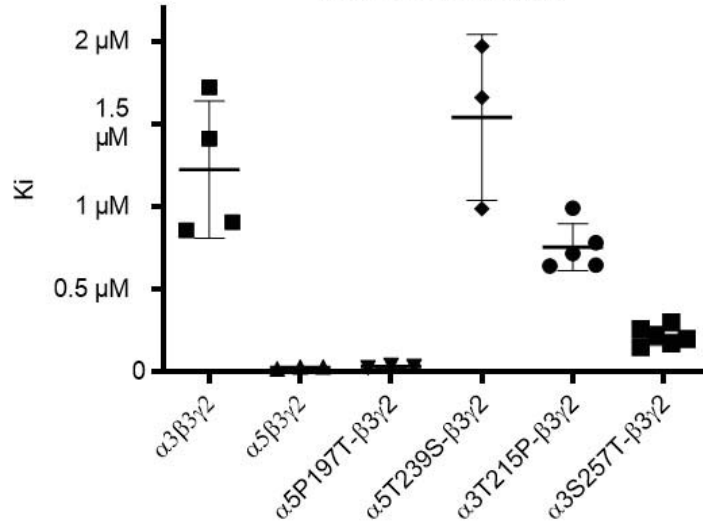
Table 2: Summary of the homologous amino acids in Loops B and C

	rat with signal peptide	rat without signal peptide	human with signal peptide	human without signal peptide
$\alpha 1$ B-loop	T 189	T 162	T 190	T 163
$\alpha 3$ B-loop	T 215	T 187	T 215	T 187
$\alpha 5$ B-loop	P 197	P 172	P 197	P 166
$\alpha 1$ C-loop	S 232	S 205	S 232	S 205
$\alpha 3$ C-loop	S 257	S 229	S 257	S 229
$\alpha 5$ C-loop	T 239	T 214	T 239	T 208

Supplemental Figure 2: Detailed statistical analyses of SH53d-acid and SH53d-ester binding to mutated receptors

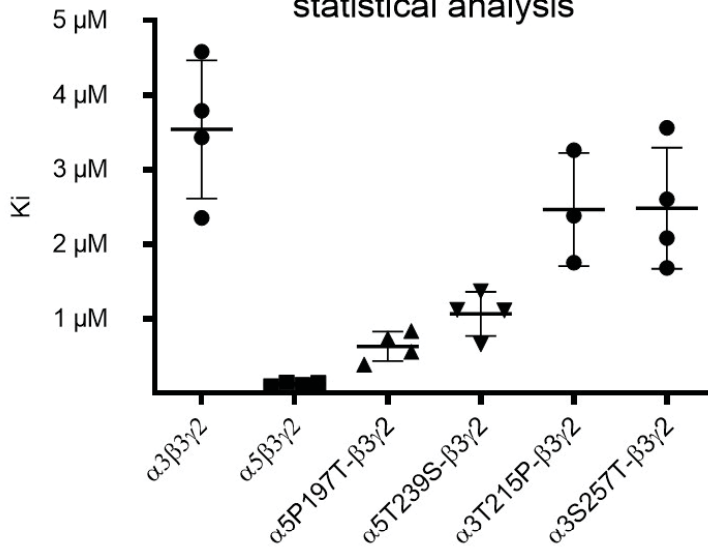
Membranes from transfected HEK 293 cells were incubated with 5 nM [³H]Ro 15-4513 in the presence of various concentrations of SH53d-acid or SH53d-ester. The concentrations resulting in half maximal inhibition of radioligand binding were converted into K_i values by using the Cheng-Prusoff relationship and the respective K_D values given in Table 3 of the main manuscript. One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 8.3.0 for Mac OS X, GraphPad Software, La Jolla, California, USA, www.graphpad.com.

SH53d-acid
statistical analysis



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
$\alpha 3\beta 3\gamma 2$ vs. $\alpha 5\beta 3\gamma 2$	1.197e-006	5.916e-007 to 1.802e-006	Yes	****	<0.0001
$\alpha 3\beta 3\gamma 2$ vs. $\alpha 5P197T-\beta 3\gamma 2$	1.191e-006	5.861e-007 to 1.796e-006	Yes	****	<0.0001
$\alpha 3\beta 3\gamma 2$ vs. $\alpha 5T239S-\beta 3\gamma 2$	-3.163e-007	-9.215e-007 to 2.888e-007	No	ns	0.5720
$\alpha 3\beta 3\gamma 2$ vs. $\alpha 3T215P-\beta 3\gamma 2$	4.684e-007	-6.312e-008 to 9.999e-007	No	ns	0.1030
$\alpha 3\beta 3\gamma 2$ vs. $\alpha 3S257T-\beta 3\gamma 2$	1.006e-006	4.949e-007 to 1.518e-006	Yes	****	<0.0001
$\alpha 5\beta 3\gamma 2$ vs. $\alpha 5P197T-\beta 3\gamma 2$	-5.500e-009	-6.524e-007 to 6.414e-007	No	ns	>0.9999
$\alpha 5\beta 3\gamma 2$ vs. $\alpha 5T239S-\beta 3\gamma 2$	-1.513e-006	-2.160e-006 to -8.662e-007	Yes	****	<0.0001
$\alpha 5\beta 3\gamma 2$ vs. $\alpha 3T215P-\beta 3\gamma 2$	-7.284e-007	-1.307e-006 to -1.498e-007	Yes	**	0.0092
$\alpha 5\beta 3\gamma 2$ vs. $\alpha 3S257T-\beta 3\gamma 2$	-1.905e-007	-7.507e-007 to 3.698e-007	No	ns	0.8829
$\alpha 5P197T-\beta 3\gamma 2$ vs. $\alpha 5T239S-\beta 3\gamma 2$	-1.508e-006	-2.155e-006 to -8.607e-007	Yes	****	<0.0001
$\alpha 5P197T-\beta 3\gamma 2$ vs. $\alpha 3T215P-\beta 3\gamma 2$	-7.229e-007	-1.302e-006 to -1.443e-007	Yes	**	0.0098
$\alpha 5P197T-\beta 3\gamma 2$ vs. $\alpha 3S257T-\beta 3\gamma 2$	-1.850e-007	-7.452e-007 to 3.753e-007	No	ns	0.8947
$\alpha 5T239S-\beta 3\gamma 2$ vs. $\alpha 3T215P-\beta 3\gamma 2$	7.847e-007	2.061e-007 to 1.363e-006	Yes	**	0.0048
$\alpha 5T239S-\beta 3\gamma 2$ vs. $\alpha 3S257T-\beta 3\gamma 2$	1.323e-006	7.624e-007 to 1.883e-006	Yes	****	<0.0001
$\alpha 3T215P-\beta 3\gamma 2$ vs. $\alpha 3S257T-\beta 3\gamma 2$	5.379e-007	5.815e-008 to 1.018e-006	Yes	*	0.0230

SH53d-ester statistical analysis



Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
$\alpha3\beta3\gamma2$ vs. $\alpha5\beta3\gamma2$	3.417e-006	2.064e-006 to 4.771e-006	Yes	****
$\alpha3\beta3\gamma2$ vs. $\alpha5P197T\text{-}\beta3\gamma2$	2.909e-006	1.555e-006 to 4.263e-006	Yes	****
$\alpha3\beta3\gamma2$ vs. $\alpha5T239S\text{-}\beta3\gamma2$	2.473e-006	1.119e-006 to 3.827e-006	Yes	***
$\alpha3\beta3\gamma2$ vs. $\alpha3T215P\text{-}\beta3\gamma2$	1.074e-006	-3.878e-007 to 2.536e-006	No	ns
$\alpha3\beta3\gamma2$ vs. $\alpha3S257T\text{-}\beta3\gamma2$	1.058e-006	-2.960e-007 to 2.411e-006	No	ns
$\alpha5\beta3\gamma2$ vs. $\alpha5P197T\text{-}\beta3\gamma2$	-5.080e-007	-1.862e-006 to 8.455e-007	No	ns
$\alpha5\beta3\gamma2$ vs. $\alpha5T239S\text{-}\beta3\gamma2$	-9.440e-007	-2.298e-006 to 4.095e-007	No	ns
$\alpha5\beta3\gamma2$ vs. $\alpha3T215P\text{-}\beta3\gamma2$	-2.343e-006	-3.805e-006 to -8.809e-007	Yes	**
$\alpha5\beta3\gamma2$ vs. $\alpha3S257T\text{-}\beta3\gamma2$	-2.360e-006	-3.713e-006 to -1.006e-006	Yes	***
$\alpha5P197T\text{-}\beta3\gamma2$ vs. $\alpha5T239S\text{-}\beta3\gamma2$	-4.360e-007	-1.790e-006 to 9.175e-007	No	ns
$\alpha5P197T\text{-}\beta3\gamma2$ vs. $\alpha3T215P\text{-}\beta3\gamma2$	-1.835e-006	-3.297e-006 to -3.729e-007	Yes	**
$\alpha5P197T\text{-}\beta3\gamma2$ vs. $\alpha3S257T\text{-}\beta3\gamma2$	-1.852e-006	-3.205e-006 to -4.980e-007	Yes	**
$\alpha5T239S\text{-}\beta3\gamma2$ vs. $\alpha3T215P\text{-}\beta3\gamma2$	-1.399e-006	-2.861e-006 to 6.312e-008	No	ns
$\alpha5T239S\text{-}\beta3\gamma2$ vs. $\alpha3S257T\text{-}\beta3\gamma2$	-1.416e-006	-2.769e-006 to -6.199e-008	Yes	*
$\alpha3T215P\text{-}\beta3\gamma2$ vs. $\alpha3S257T\text{-}\beta3\gamma2$	-1.667e-008	-1.479e-006 to 1.445e-006	No	ns