Targeting nitric oxide production in microglia with novel imidazodiazepines for non-sedative pain treatment

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Contents

Table S1. Compound list with references

GL-I-48[1-3]	GL-II-05[4]	GL-III-13[5-7]	GL-III-70[8]	GL-IV-01[8]	DMH-D-053[1-3]
GL-I-50[1, 2]	GL-II-06[2]	GL-III-23[8]	GL-III-72[5]	GL-IV-03[8]	KRM-II-18B[5, 6, 9]
GL-I-62[10]	GL-II-18[2, 8, 11]	GL-III-24[10]	GL-III-73[5]	GL-IV-04[8]	KRM-II-81[5, 6, 9, 12-14]
GL-I-64[10]	GL-II-19[2, 8, 11]	GL-III-25[10]	GL-III-75	GL-IV-05[15]	KRM-II-82[5, 6]
GL-I-65[8]	GL-II-32	GL-III-27[10]	GL-III-76[5]	GL-IV-17[8, 11, 16]	MP-III-022[8]
GL-I-66[8]	GL-II-33[8]	GL-III-35	GL-III-76A[5]	GL-IV-18[11, 17]	MP-III-080[5, 6, 8, 12]
GL-I-81[8]	GL-II-51[11]	GL-III-36[5]	GL-III-77[5]		MP-IV-004[8]
	GL-II-54[8]	GL-III-42[11]	GL-III-78[5]		MP-IV-005[8]
	GL-II-61[10]	GL-III-52[18]	GL-III-84		MP-IV-010[8]
	GL-II-79[5]	GL-III-53[11]	GL-III-85		QH-II-66[19]
	GL-II-80[5]	GL-III-54[11]	GL-III-86		YT-III-271[1-3]
		GL-III-58[20]	GL-III-87		YT-III-31[5, 7, 21]
		GL-III-59[20]	GL-III-97[8]		
		GL-III-60[22] GL-III-63[8]	GL-III-98[8]		DMH-D-053[1-3]
		GL-III-64[8]			KRM-II-18B[5, 6, 9]
		GL-III-66[8]			KRM-II-81[5, 6, 9, 12-14]
		GL-III-67[8]			KRM-II-82[5, 6]
		GL-III-68[8]			
		GL-III-69[8]			

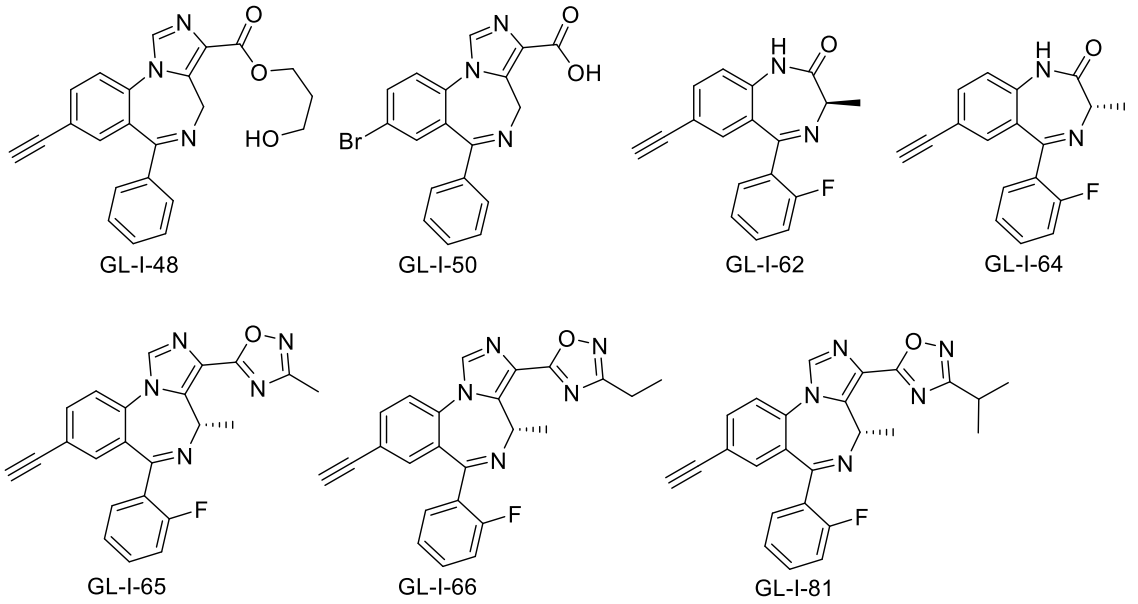

Figure S1. GL-I compounds

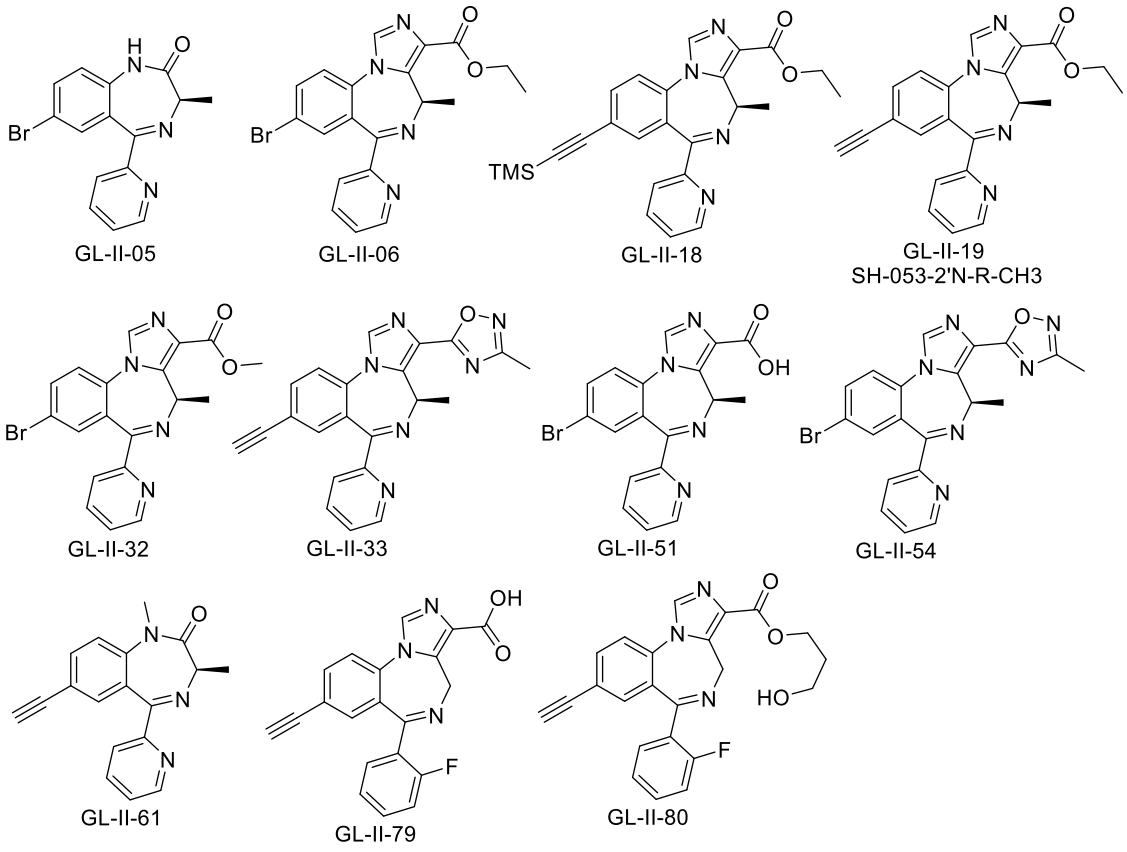

Figure S2. GL-II compounds

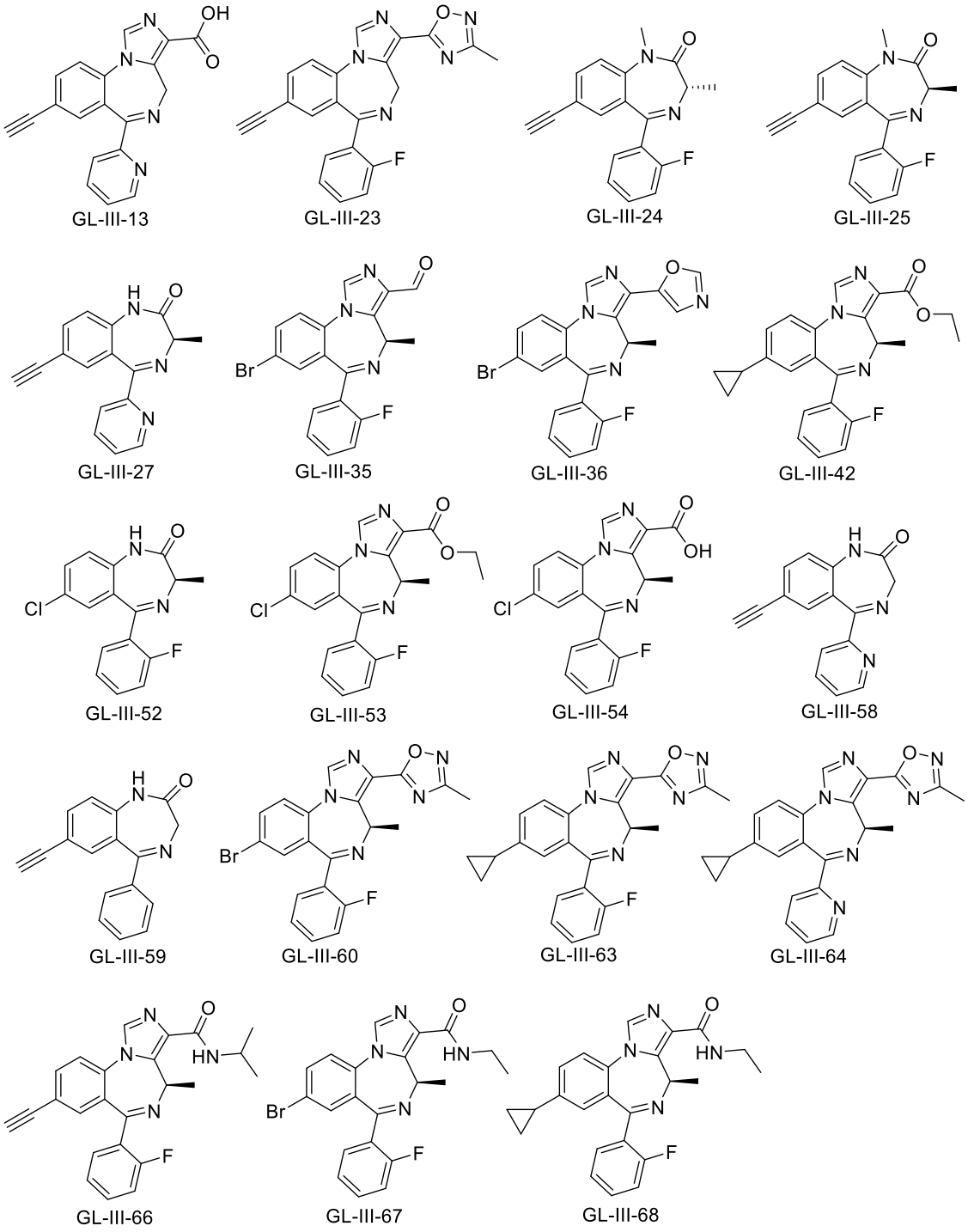

Figure S3. GL-III compounds part A

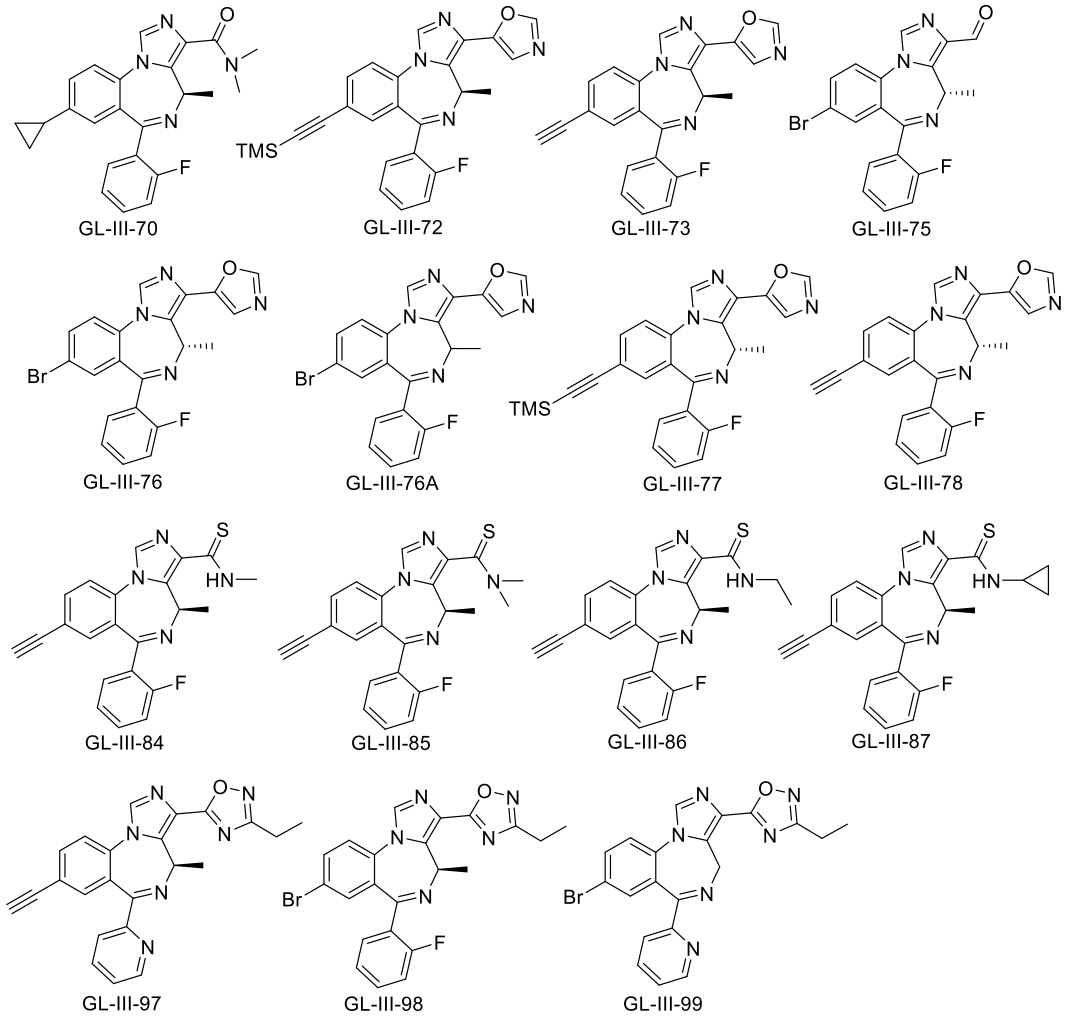

Figure S4. GL-III compounds part B

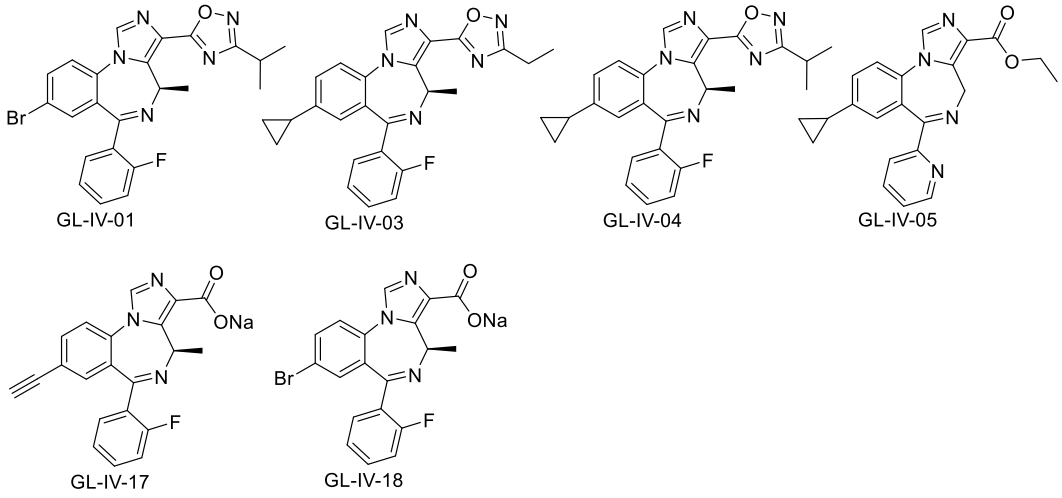

Figure S5. GL-IV compounds

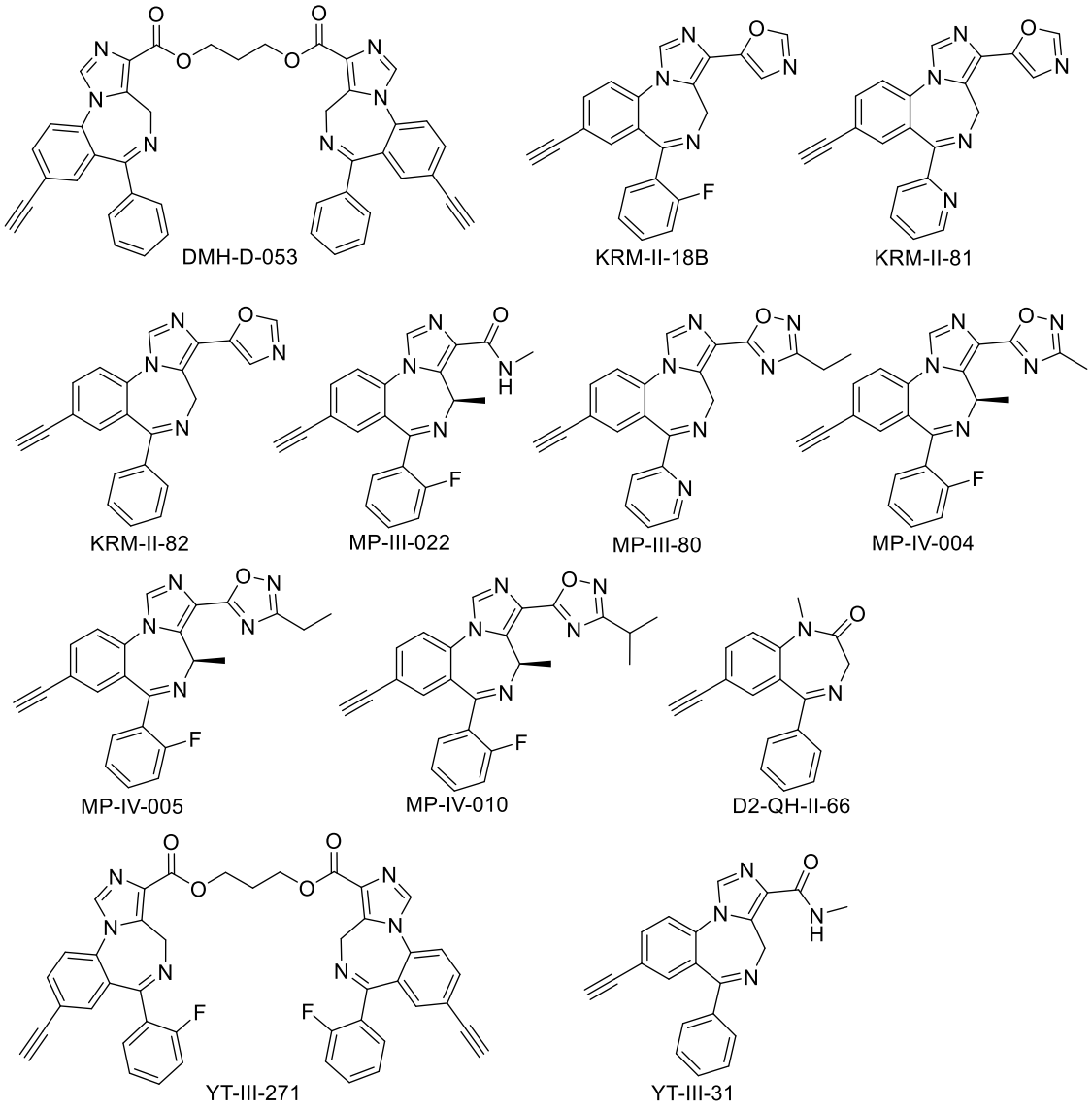

Figure S6. Various compounds

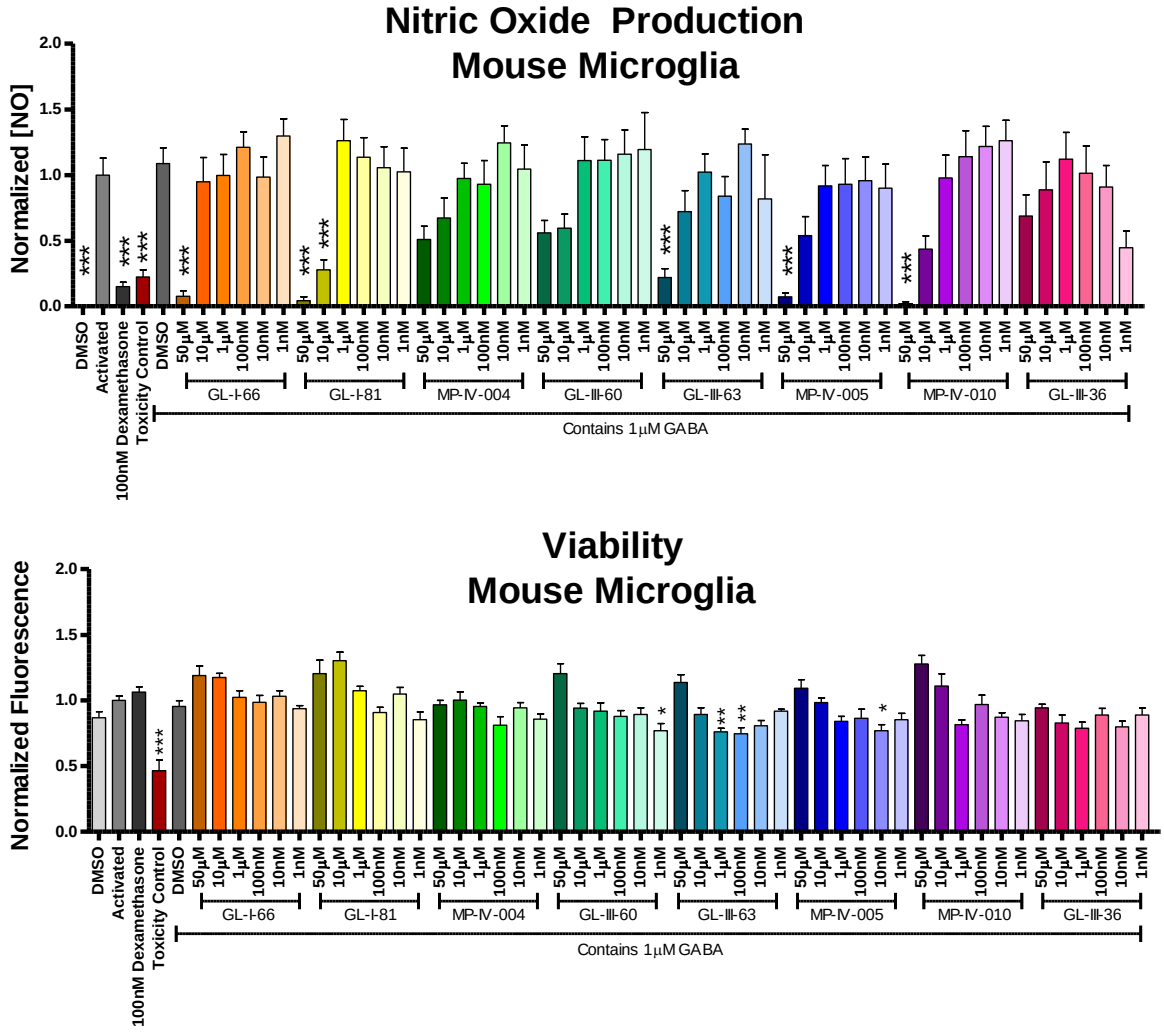

Figure S7. Concentration response of compounds with significant reduction of NO production for mouse microglia. A) Cells were activated with LPS and IFNγ and treated with 1 µM GABA and - 1 nM - 50 µM of compound for 24 hours. NO in supernatant was quantified with a Griess assay. B) ATP in remaining cells was quantified by the Cell Titer Glo as a measure of viability. Means ± SEM are presented for n = 12. *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. (ANOVA)

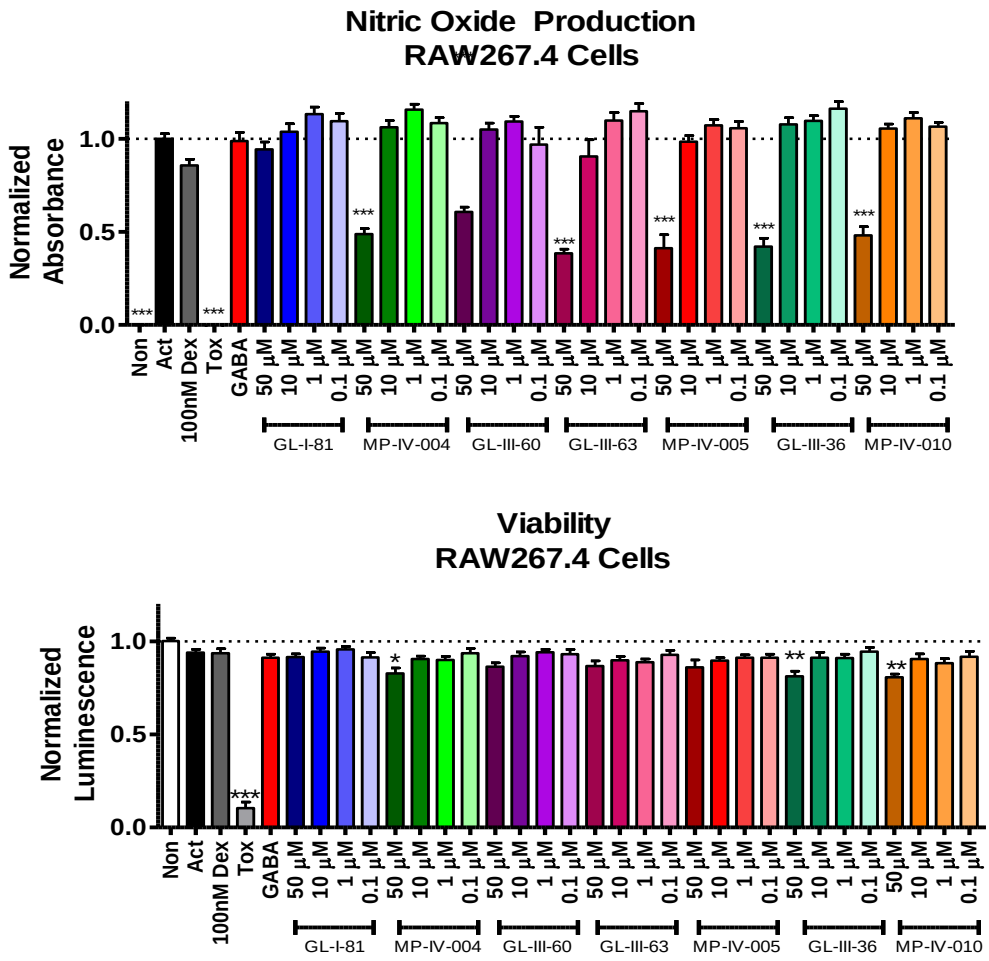

Figure S8. Concentration response of compounds with significant reduction of NO production for mouse macrophages (RAW267.4). A) Cells were activated with LPS and IFNγ and treated with 1 µM GABA and 100 nM - 50 µM of compound for 24 hours. NO in supernatant was quantified with a Griess assay. B) ATP in remaining cells was quantified by the Cell Titer Glo as a measure of viability. Means ± SEM are presented for n = 12. *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. (ANOVA)

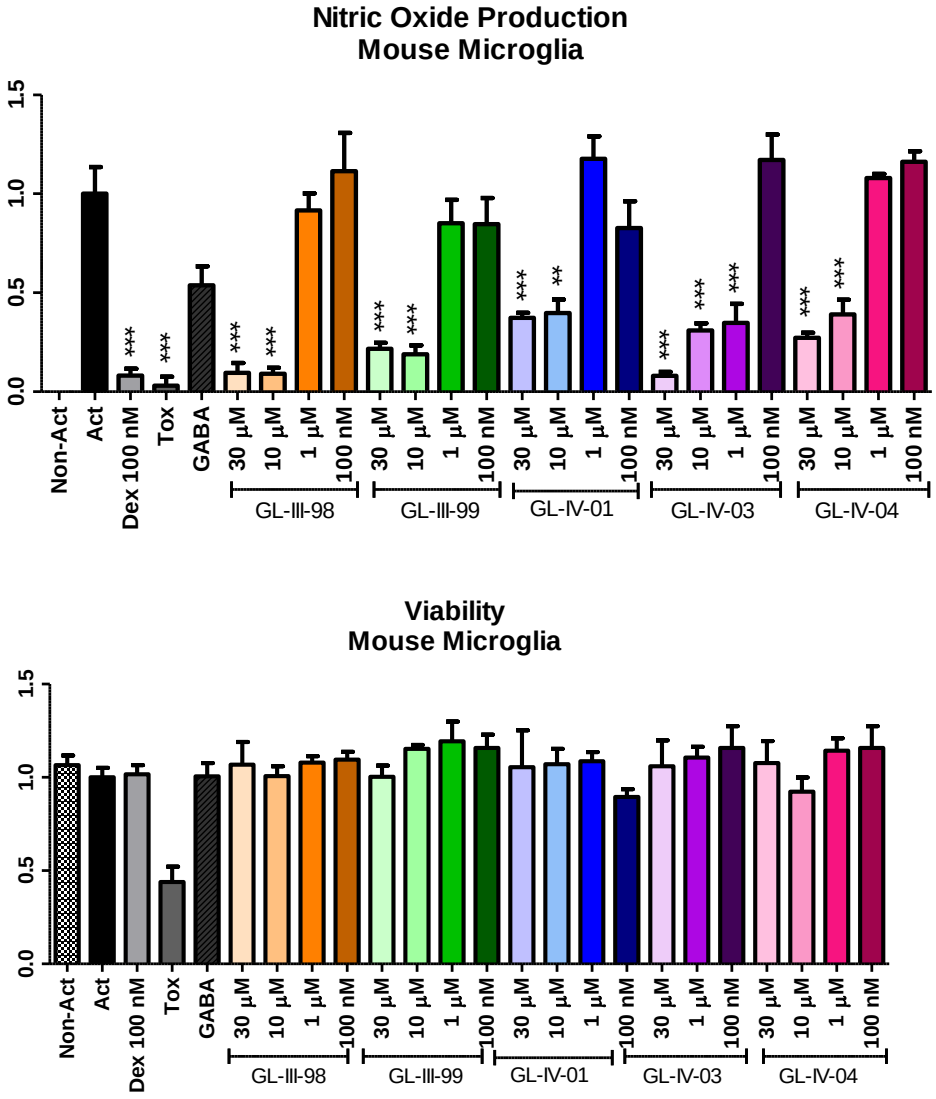

Figure S9. Concentration response of compounds with significant reduction of NO production for mouse microglia. A) Cells were activated with LPS and IFNγ and treated with 1 µM GABA and 100 nM - 30 µM of compound for 24 hours. NO in supernatant was quantified with a Griess assay. B) ATP in remaining cells was quantified by the Cell Titer Glo as a measure of viability. Means ± SEM are presented for n = 12. *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. (ANOVA)

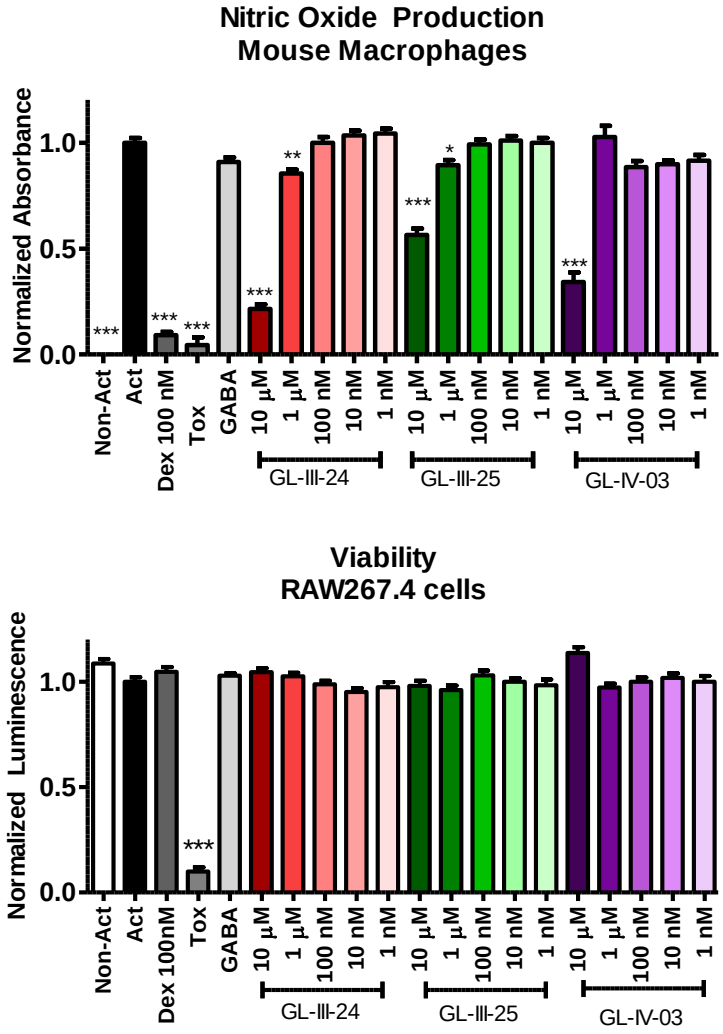

Figure S10. Concentration response of compounds with significant reduction of NO production for mouse macrophages (RAW267.4). A) Cells were activated with LPS and IFNγ and treated with 1 µM GABA and 1 nM - 10 µM of compound for 24 hours. NO in supernatant was quantified with a Griess assay. B) ATP in remaining cells was quantified by the Cell Titer Glo as a measure of viability. Means ± SEM are presented for n = 12. *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. (ANOVA)

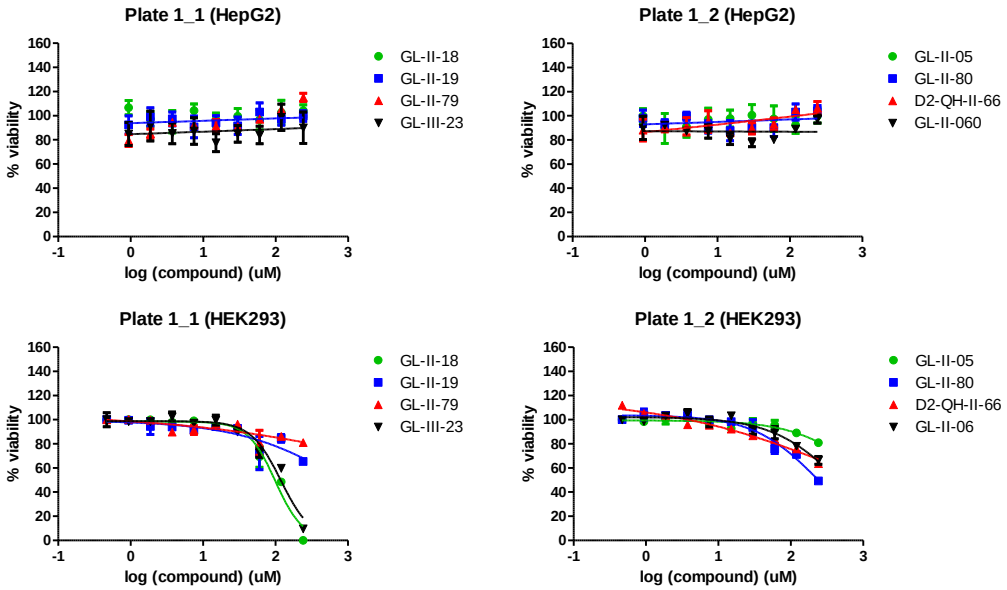

Figure S11. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 1. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

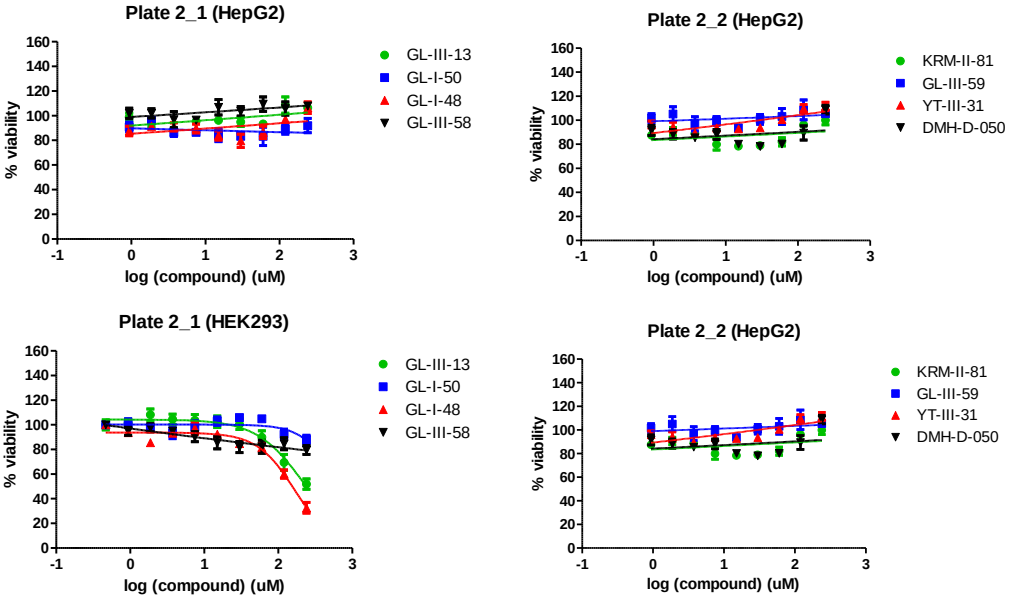

Figure S12. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 2. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

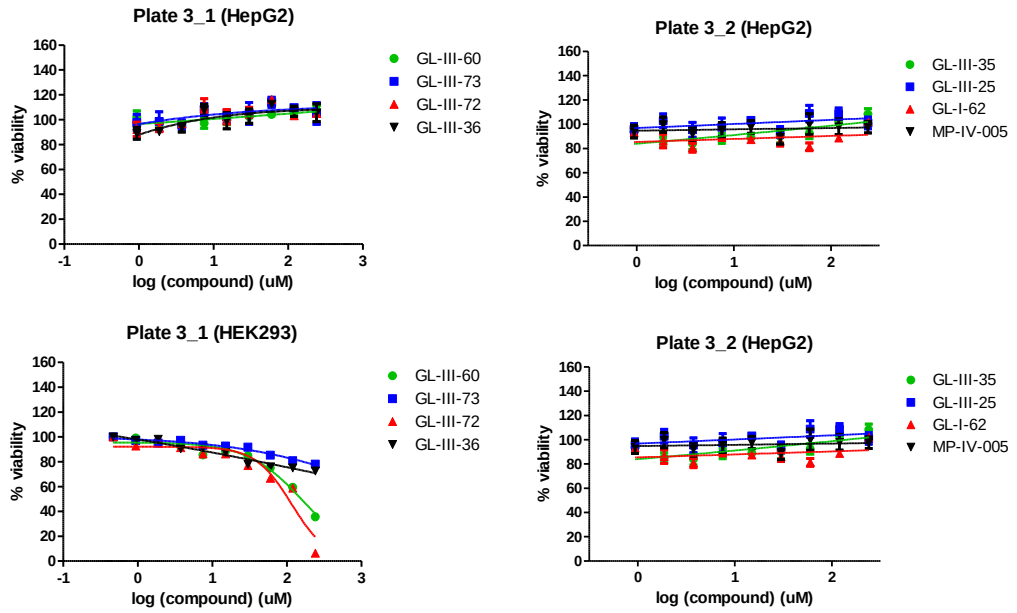

Figure S13. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 3. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

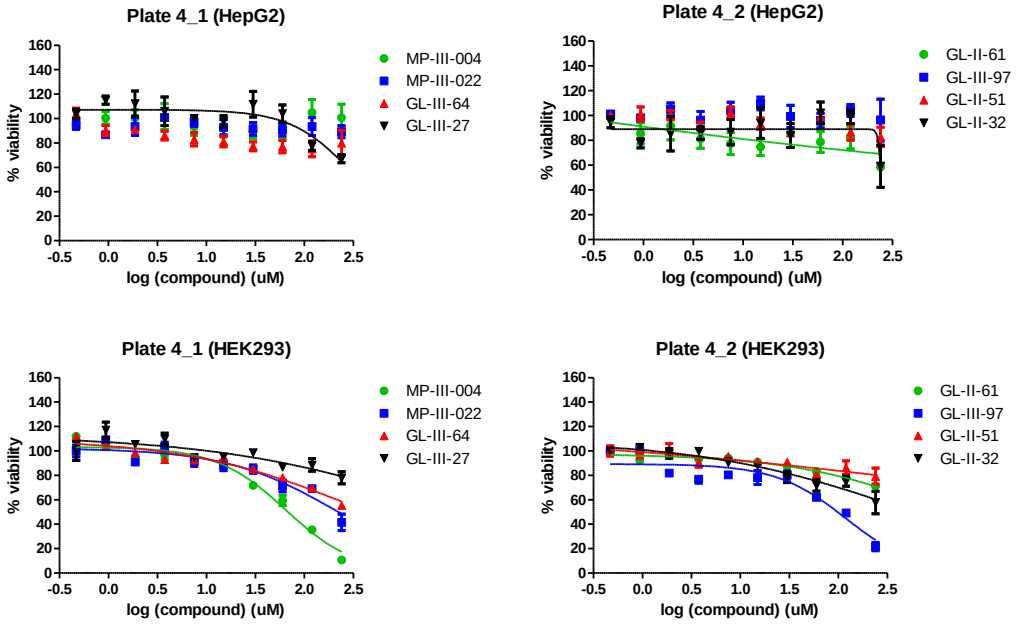

Figure S14. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 4. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

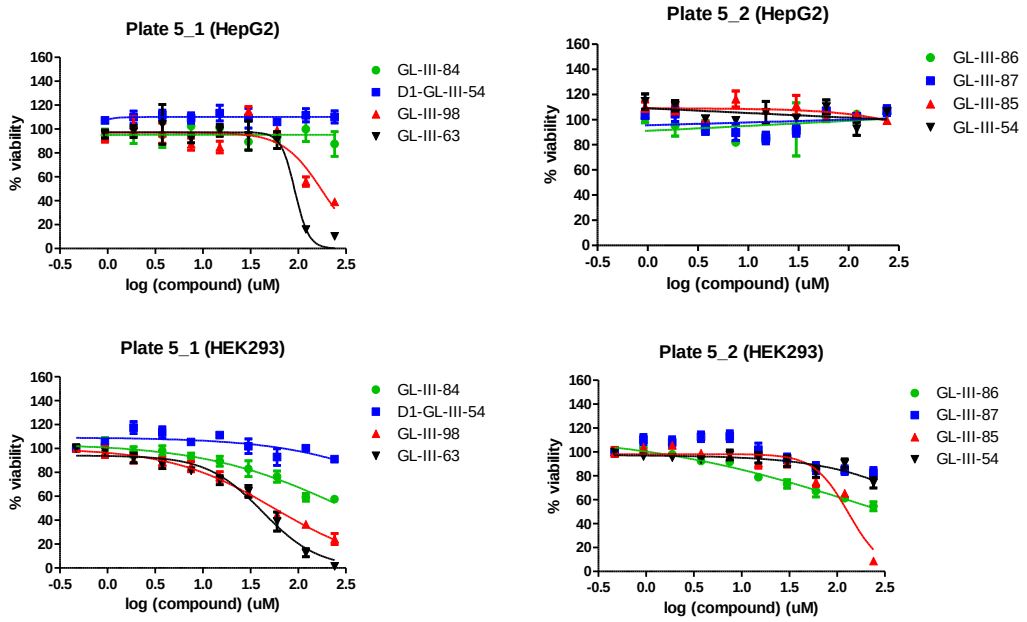

Figure S15. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 5. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

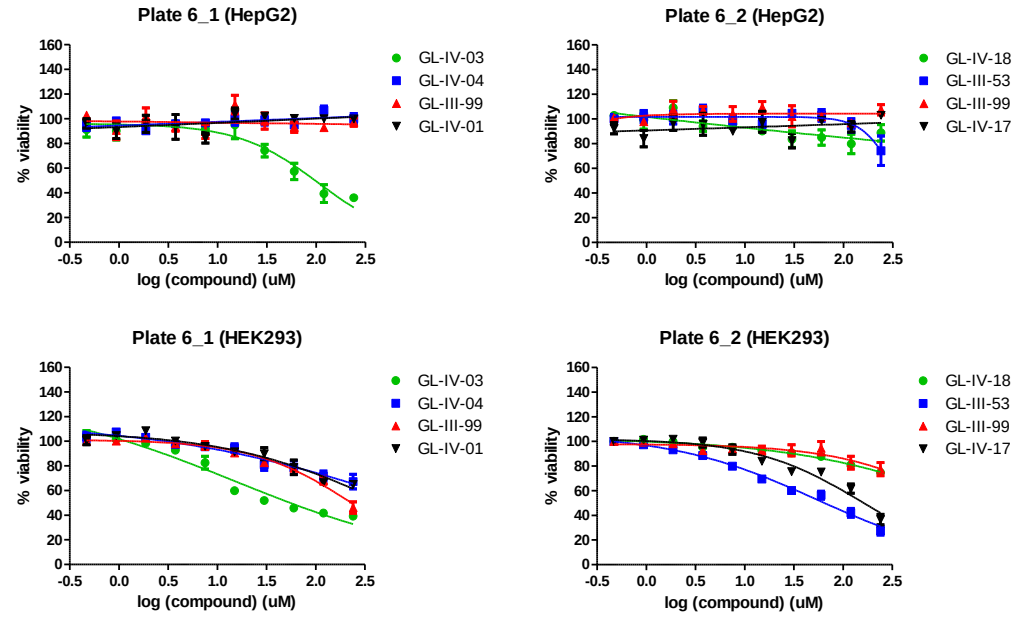

Figure S16. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 6. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

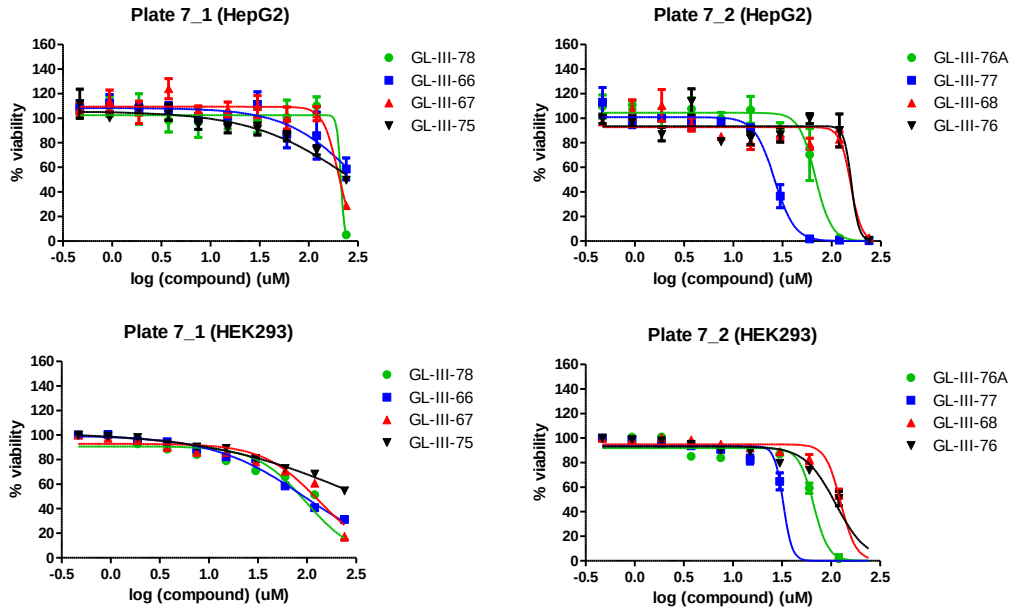

Figure S17. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 7. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

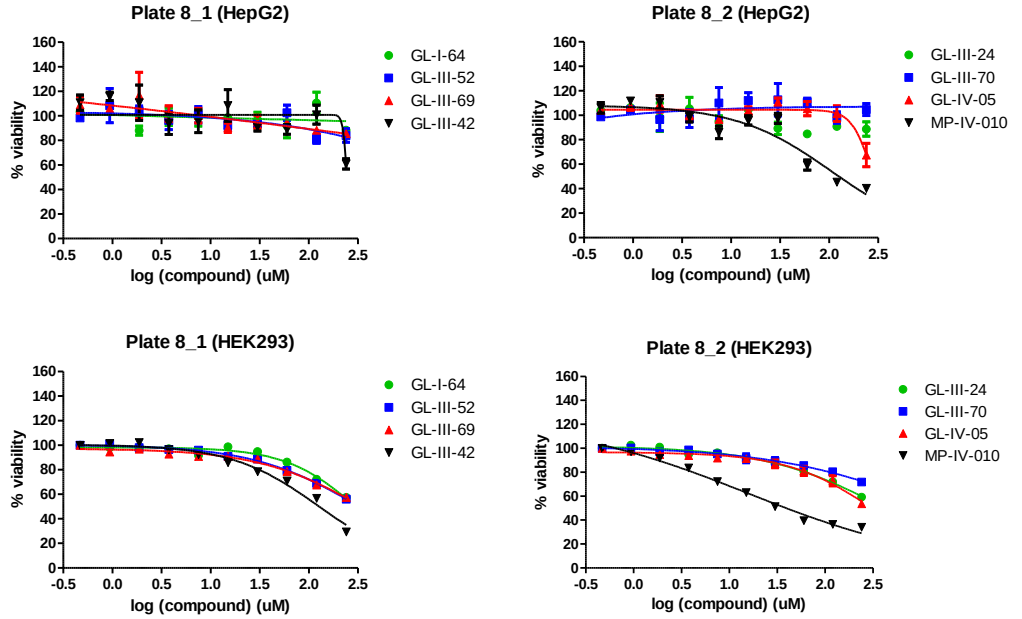

Figure S18. Concentration dependent viability of HepG2 (liver) and HEK293 (kidney) in the presence of compounds from plate 8. Cells were dispensed into 384 well plates and treated with different concentrations of compounds for 24 hours. The cell viability was determined with CellTiter-Glo. (n = 8 for each concentration)

Table S2. PDSP Primary Screening Data for MP-IV-010.

Receptor	Percent Competition	Receptor	Percent Competition	Receptor	Percent Competition
5-HT1A	19	**D5**	29	**Alpha2A**	14
5-HT1B	29	**SERT**	22	**Alpha2B**	29
5-HT1D	6	**NET**	-2	**Alpha2C**	31
5-HT1E	-7	**DAT**	14	**Beta1**	6
5-HT2A	-1	**MOR**	33	**Beta2**	20
5-HT2B	0	**DOR**	5	**M1**	-11
5-HT2C	-2	**KOR**	94	**M2**	10
5-HT3	13	**GABAA**	-8	**M3**	0
5-HT5A	11	**H1**	19	**M4**	8
5-HT6	21	**H2**	28	**M5**	26
5-HT7	-5	**H3**	-18	**Beta3**	38
D1	28	**H4**	6	**BZP Rat Brain Site**	64
D2	25	**Calcium Channel**	15	**PBR**	28
D3	15	**Alpha1A**	2	**Alpha1D**	9
D4	5	**Alpha1B**	15	**HERG binding**	29
				Sigma 2	64

Table S3. Human $GABA_AR$ Subunit PCR Primers

$GABA_A$ R Subunit	**Forward**	**Reverse**	**Size**	**Ref**
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452	[23]
α1	GGA TTG GGA GAG CGT GTA ACC	TGA AAC GGG TCC GAA ACT G	66	[23]
α2	GTT CAA GCT GAA TGC CCA AT	ACC TAG AGC CAT CAG GAG CA	160	[23]
α3	CAA CTT GTT TCA GTT CAT TCA TCC TT	CTT GTT TGT GTG ATT ATC ATC TTC TTA GG	102	[23]
α4	TTG GGG GTC CTG TTA CAG AAG	TCT GCC TGA AGA ACA CAT CCA	105	[23]
α5	CTT CTC GGC GCT GAT AGA GT	CGC TTT TTC TTG ATC TTG GC	105	[23]
α6	CTG AAC CTT TGG AAG CTG AGA	TTA TTG GCC TCG GAA GAT GA	109	[24]
β1	CCA GGT CGA CGC CCA CGG TA	GTG GCC TTG GGG TCG CTC AC	102	[23]
β2	GCA GAG TGT CAA TGA CCC TAG T	GGC AAT GTC AAT GTT CAT CCC	137	[23]
β3	CCG TTC AAA GAG CGA AAG CAA CCG	TCG CCA ATG CCG CCT GAG AC	105	[23]
γ1	CCT TTT CTT CTG CGG AGT CAA	CAT CTG CCT TAT CAA CAC AGT TTC C	91	[23]
γ2	CAC AGA AAA TGA CGG TGT GG	TCA CCC TCA GGA ACT TTT GG	136	[23]
γ3	AAC CAA CCA CCA CGA AGA AGA	CCT CAT GTC CAG GAG GGA AT	131	[23]
δ	GAG GCC AAC ATG GAG TAC AC	TTC ACG ATG AAG GTG TCG G	145	[25]

Table S4. Mouse $GABA_AR$ Subunit PCR Primers

$GABA_AR$ Subunit	Forward	Reverse	Size	Ref
α1	CAA GAG CAG AAG TTG TCT ATG AGT	GCA CGG CAG ATA TGT TTG AAT AAC	215	[26]
α2	GCT ACG CTT ACA CAA CCT CAG A	GAC TGG CCC AGC AAA TCA TAC T	115	[26]
α3	GCC GTC TGT TAT GCC TTT GTA TTT	TTC TTC ATC TCC AGG GCC TCT	119	[27]
α4	AGA ACT CAA AGG ACG AGA AAT TGT	TTC ACT TCT GTA ACA GGA CCC C	118	[23]
α5	AAG TTC GCT CCG GCA GTA TG	TGT TCT TGC CTC CAA CTT GAT CT	149	[27]
α6	CTT GCT GGA AGG CTA TGA CAA C	AAG TCT GGC GGA AGA AAA CAT C	146	[27]
β1	GGT TTG TTG TGC ACA CAG CTC C	ATG CTG GCG ACA TCG ATC CGC	153	[23]
β2	GCT GGT GAG GAA ATC TCG GTC CC	CAT GCG CAC GGC GTA CCA AA	70	[23]
β3	CTT TGC GGG AGG AAG GCT TT	GGG GTC GTT TAC GCT CTG AG	85	[27]
γ1	ATC CAC TCT CAT TCC CAT GAA CAG C	ACA GAA AAA GCT AGT ACA GTC TTT GC	100	[23]
γ2	ACT TCT GGT GAC TAT GTG GTG AT	GGC AGG AAC AGC ATC CTT ATT G	147	[26]
γ3	ATT ACA TCC AGA TTC CAC AAG ATG	CAC AGG TGT CCT CAA ATT CCT	149	[23]
δ	TCA AAT CGG CTG GCC AGT TCC C	GCA CGG CTG CCT GGC TAA TCC	147	[28]
GAPDH	AAC ACA GTC CAT GCC ATC AC	CAC CAC CCT GTT GCT GTA GCC	450	[23]

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