Screening for New Inhibitors of Glycine Transporter 1 and 2 by Means of MS Binding Assays

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A straightforward screening of a compound library comprising 2439 substances for the identification of new inhibitors for the neurotransmitter transporters GlyT1 and GlyT2 is described. Screening and full-scale competition experiments were performed using recently developed GlyT1 and GlyT2 MS Binding Assays. That way for both targets, GlyT1 and GlyT2, ligands were identified, which exhibited affinities (pK_i values) in the low micromolar to sub-micromolar range. The majority of these binders exhibit new chemical scaffolds in the class of GlyT1 and GlyT2 inhibitors, which could be of interest for the development of new ligands with improved affinities for the target proteins. Additionally, compounds with excellent fluorescent properties were found for GlyT2, which renders them promising compounds for future fluorescence-based techniques. All in all, this study demonstrates that MS Binding Assays represent a powerful technology platform also well suited for the screening of compound libraries in a highly reliable and effective manner.

Introduction

Glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2) are members of the Na^+/Cl^--dependent solute carrier 6 (SLC6) family which serve to terminate the signaling of the neurotransmitter glycine by mediating its reuptake from the synaptic cleft in the central nervous system. Of these two glycine transporters, GlyT1 is found on glial cells surrounding inhibitory glycinergic neurons as well as on pre- and postsynaptic terminals of excitatory glutamatergic neurons and adjoining glial cells, whereas GlyT2 is only present at presynaptic terminals of glycinergic neurons.[1–3] At glycinergic neurons neurotransmission is characterized by glycine release from the presynaptic neuron into the synaptic cleft and glycine binding toward glycine receptors (GlyR) on the postsynaptic neuron, which leads to a hyperpolarization due to an Cl^- inward current. At these neurons glycine signaling is terminated by reuptake of glycine from the synaptic cleft by GlyT1 and GlyT2 into the glial cells and presynaptic neuron, respectively.[1,3] Thereby, GlyT2 is important for the recycling of glycine, as the neurotransmitter by transport into the presynaptic glycinergic neuron, can be reused for signaling.[3] At glutamatergic neurons glycine concentration in the synaptic cleft is regulated by GlyT1 only, present on the pre- and postsynaptic neurons and glial cells. There it acts as a co-agonist of the neurotransmitter L-glutamate at NMDA (*N*-methyl-D-aspartate) receptors. Glycine binding at this so-called strychnine-insensitive glycine-B binding site of the NMDA receptor, enhances excitability of the latter, as it increases the affinity of L-glutamate by a positive allosteric effect.[4]

Due to their presence at glycinergic and glutamatergic neurons GlyT1 and GlyT2 found widespread interest as drug targets during the last years. Both appear to possess a high potential as drug targets for the treatment of diseases related to a deficiency in glycine or glutamate signaling such as drug addiction (e.g. alcohol dependence),[5–12] neuropathic chronic pain[13–20] or negative and cognitive symptoms of schizophrenia,[21–25] for which no appropriate therapies are available so far. For several drug candidates positive results in preclinical animal studies were found for the mentioned diseases.[2,13] Over the years several of these compounds, in particular GlyT1 inhibitors, were even introduced into clinical trials.[2,26] At the moment, clinical trials for three GlyT1 and GlyT2 inhibitors are in progress. The GlyT1 inhibitors PF-03463275 and BI 425809 are under investigation for the treatment of cognitive impairment associated with schizophrenia[27,28] and the latter, additionally, for the treatment of cognitive impairment in Alzheimer's disease.[29] Compound VVZ-149, representing a GlyT2 inhibitor, which in addition acts at the purine P2X3 and the serotonin 5-HT_{2A} receptors as antagonist, is under investigation for the treatment of postoperative pain.[30,31] Accordingly, there is ongoing interest in GlyT1 and GlyT2 inhibitors as potential remedies for the treatment of diseases like schizophrenia or neuropathic pain lacking a suitable medication, so far.

The aim of the present study was to identify new inhibitors of the glycine transporters GlyT1 and GlyT2 exhibiting reasonable to good affinities, i.e. in the low micromolar to sub-micromolar (pK_i) range. To this end, a set of about 2400 compounds available to us from former studies should be screened in competitive binding assays for their affinities toward these transporters. For this screening, the recently published GlyT1 and GlyT2 MS Binding Assays based on the

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selective GlyT1 and GlyT2 inhibitors Org24598 and Org25543, respectively, as reporter ligands should be used (see Figure 1).[32,33] Of the aforementioned compound library those compounds, representing α-amino acids, should be studied individually, all other screened in sublibraries consisting of 16 compounds. In case of the individually studied compounds, hits would, of course, become directly apparent. Sublibraries identified as active should be subjected to deconvolution by testing the individual compounds. Finally, the whole screening process should be complemented by determination of the equilibrium dissociation constants (*K*i) of the hit compounds in competition experiments, to verify and fully characterize the individual hits.

Results and Discussion

Compound library set-up and screening procedure

The compound library that should be searched for new GlyT inhibitors consists of 2439 compounds in total. Most of these compounds had been synthesized in our group aiming at the identification and development of new inhibitors for different neurotransmitter transporters of the SLC6 transporter family. Additionally, to some extent, precursors, intermediates, and side products of synthetic routes were included. The molecular weights of the compounds we tested range from 60–1290 Da. Of these compounds the molecular weight of 99% is in between 60–600 Da and 91% do not surpass 500 Da.

For the screening procedure we decided, as already mentioned above, to divide the compound library into two subsets, one in which compounds should be tested individually, and another one in which they should be studied as sublibraries each comprising 16 constituents. With glycine as a substrate for GlyT1 and GlyT2, we expected compounds with an α-amino acid subunit to show a relatively high hit rate. For this reason, it seemed more efficient to test these compounds individually. Hence, 170 compounds (α-amino acids) were tested as single compounds (**1**–**170**), whereas 2269 compounds were studied in form of 141 sublibraries each containing 16 and one sublibrary containing 13 constituents (**Sublibrary 1**–**Sublibrary 142**).

For the screening procedure we used the recently described GlyT1 and GlyT2 MS Binding Assays.[32,33] As we aimed at the identification of compounds with an binding affinity expressed as IC_{50} of at least 10 μM (for GlyT1 and for GlyT2) the α-amino acids and sublibraries should first be studied at the concentration of 10 μM. In case, a large series of the tested compounds and/or sublibraries should meet this selection criterion, these entities should be subjected to a second screening round, with a more stringent selection criterion. In this second screening round the compounds and sublibraries identified as active in the first screening process (reduction of specific marker binding to ≤50% for Gly1 and GlyT2, respectively, at a test compound concentration of 10 μM) should then be studied at a concentration of 1.0 μM. In the event, this second more stringent screening process (test compound concentration 1.0 μM) were to be performed and led to the identification of active entities (reduction of specific marker binding to ≤50%) the latter should be subjected to further analysis, otherwise, studies should continue with the active entities found in the first screening round (at a test compound concentration of 10 μM).

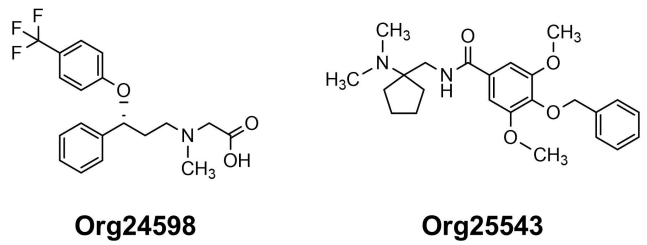

Figure 1. Structures of Org24598 and Org25543 used as reporter ligands in the GlyT1 and GlyT2 MS Binding Assays, respectively.

For single compounds that had fulfilled the selection criteria, in full scale competition experiments the IC_{50} value and from there the pK_i should be determined. In case of active sublibraries deconvolution experiments should be performed to identify the hit compounds (fulfilling selection criterion, i.e., reduction of specific marker binding to ≤50% for GlyT1 and GlyT2, respectively, at the respective test compound concentration) for which then as for the single compounds the pK_i values should be determined.

For the sake of clarity, it is to be stated here that the sublibrary constituents are denominated as **SCx**–**y** with x referring to the sublibrary and y to the individual sublibrary constituent **a**–**p** in this library.

Compound library screening for GlyT1

During the 10 μM screening for GlyT1 eight α-amino acids and 89 sublibraries were found to reduce specific marker binding to ≤50% and were thus qualified as active (see Table 1). As described before, the 89 sublibraries as well as the eight individual compounds fulfilling the selection criterion should be again studied for their binding affinity for GlyT1 at a concentration of 1.0 μM. However, the following screening round was continued only with the compounds and sublibraries which reduced the specific marker binding to ≤25%. This decision was made due to the high number of active α-amino acids and sublibraries and since it was expected that those with specific marker binding reduction of >25% would not be found as active anymore at a concentration of 1.0 μM. Therefore, the second screening round was continued with the two α-amino acids **47** and **48** as well as 40 sublibraries. At this concentration level one compound, **48** (see Figure 2), and five sublibraries, sublibraries 73, 116, 120, 121 and 140, were found to reduce specific marker binding to ≤50% (for **48** to 18.2±1.6% and for sublibrary 73 to 46.7±7.8%, for sublibrary 116 to 48.1±1.9%, for sublibrary 120 to 48.8±4.1%, for sublibrary 121 to 11.7±2.0% and for sublibrary 140 to 34.0±3.5%; see Table 1). When the single constituents of the five active sublibraries were tested at a concentration of 1.0 μM in deconvolution experiment only four test compounds contained in sublibraries 121

Table 1. Results of the 10 μM and 1.0 μM GlyT1 MS Binding Assay screening for the α-amino acids and sublibraries fulfilling the selection criteria.

Compound/ Sublibrary	Remaining specific binding of Org24598 at **10 μM** [%][a]	Remaining specific binding of Org24598 at **1.0 μM** [%][a]	Compound/ Sublibrary	Remaining specific binding of Org24598 at **10 μM** [%][a]	Remaining specific binding of Org24598 at **1.0 μM** [%][a]
1	27.8 ± 4.2	–	**Sublibrary 70**	27.0 ± 4.8	–
2	43.7 ± 14.7	–	***Sublibrary 73***	3.4 ± 5.1	*46.7 ± 7.8*
30	42.0 ± 4.3	–	**Sublibrary 74**	16.3 ± 5.4	80.5 ± 4.4
47	7.0 ± 1.0	57.0 ± 6.2	**Sublibrary 75**	17.0 ± 2.7	78.6 ± 0.4
48	2.7 ± 0.8	*18.2 ± 1.6*	**Sublibrary 76**	12.0 ± 1.8	85.7 ± 3.5
131	28.3 ± 0.6	–	**Sublibrary 77**	14.0 ± 6.6	83.2 ± 9.4
149	45.3 ± 3.6	–	**Sublibrary 78**	30.3 ± 1.0	–
165	39.0 ± 9.8	–	**Sublibrary 79**	35.9 ± 3.3	–
Sublibrary 3	39.6 ± 4.7	–	**Sublibrary 81**	49.2 ± 2.1	–
Sublibrary 11	39.4 ± 8.1	–	**Sublibrary 83**	32.3 ± 6.0	–
Sublibrary 12	38.9 ± 3.1	–	**Sublibrary 84**	26.0 ± 0.7	–
Sublibrary 13	48.6 ± 11.5	–	**Sublibrary 88**	35.8 ± 6.7	–
Sublibrary 14	43.7 ± 9.8	–	**Sublibrary 93**	31.8 ± 5.6	–
Sublibrary 15	31.2 ± 5.0	–	**Sublibrary 94**	33.6 ± 1.2	–
Sublibrary 18	29.7 ± 1.3	–	**Sublibrary 95**	18.8 ± 3.5	59.7 ± 4.0
Sublibrary 20	44.6 ± 5.0	–	**Sublibrary 96**	20.0 ± 3.9	59.1 ± 3.7
Sublibrary 26	29.4 ± 2.3	–	**Sublibrary 97**	31.1 ± 3.2	–
Sublibrary 27	39.1 ± 1.5	–	**Sublibrary 98**	33.8 ± 1.2	–
Sublibrary 28	39.8 ± 2.7	–	**Sublibrary 99**	13.8 ± 2.1	65.6 ± 7.7
Sublibrary 29	24.8 ± 7.6	75.4 ± 7.1	**Sublibrary 100**	46.2 ± 3.5	–
Sublibrary 30	13.1 ± 1.5	58.8 ± 4.6	**Sublibrary 101**	19.4 ± 4.9	73.1 ± 6.6
Sublibrary 32	33.7 ± 6.1	–	**Sublibrary 102**	18.9 ± 6.5	93.3 ± 11.3
Sublibrary 33	19.5 ± 3.5	67.4 ± 2.0	**Sublibrary 103**	41.9 ± 8.9	–
Sublibrary 34	19.1 ± 0.8	60.3 ± 8.7	**Sublibrary 104**	13.0 ± 5.7	75.6 ± 3.5
Sublibrary 35	0.9 ± 2.0	93.3 ± 5.4	**Sublibrary 105**	33.0 ± 5.1	–
Sublibrary 36	23.6 ± 0.8	70.1 ± 7.6	**Sublibrary 107**	48.4 ± 1.4	–
Sublibrary 37	12.1 ± 0.9	88.8 ± 10.5	**Sublibrary 115**	33.4 ± 2.5	–
Sublibrary 38	-1.0 ± 0.7	54.7 ± 2.5	***Sublibrary 116***	24.4 ± 2.4	*48.1 ± 1.9*
Sublibrary 39	0.5 ± 1.6	60.3 ± 4.7	**Sublibrary 117**	48.0 ± 13.2	–
Sublibrary 40	14.7 ± 4.7	77.7 ± 3.5	**Sublibrary 118**	40.9 ± 10.0	–
Sublibrary 41	8.1 ± 2.8	88.6 ± 4.6	**Sublibrary 119**	45.1 ± 2.3	–
Sublibrary 42	19.1 ± 5.9	101.0 ± 4.7	***Sublibrary 120***	2.5 ± 1.2	*48.8 ± 4.1*
Sublibrary 43	22.9 ± 1.6	95.9 ± 11.7	***Sublibrary 121***	0.9 ± 0.9	*11.7 ± 2.0*
Sublibrary 44	27.0 ± 3.3	–	**Sublibrary 122**	38.1 ± 4.5	–
Sublibrary 45	21.0 ± 1.3	96.1 ± 6.5	**Sublibrary 124**	29.6 ± 1.3	–
Sublibrary 48	30.1 ± 2.2	–	**Sublibrary 125**	47.7 ± 3.4	–
Sublibrary 49	20.6 ± 2.5	109.7 ± 8.6	**Sublibrary 126**	18.7 ± 2.1	64.3 ± 8.8
Sublibrary 50	20.0 ± 3.2	99.1 ± 2.1	**Sublibrary 127**	36.6 ± 6.6	–
Sublibrary 51	15.9 ± 2.0	81.0 ± 4.7	**Sublibrary 128**	26.3 ± 2.1	–
Sublibrary 55	37.1 ± 1.7	–	**Sublibrary 129**	34.2 ± 16.3	–
Sublibrary 56	34.7 ± 6.5	–	**Sublibrary 130**	37.5 ± 0.3	–
Sublibrary 57	13.5 ± 5.2	82.8 ± 3.2	**Sublibrary 131**	28.8 ± 7.5	–
Sublibrary 58	39.6 ± 6.4	–	**Sublibrary 132**	18.6 ± 1.6	70.3 ± 5.7
Sublibrary 59	38.6 ± 5.7	–	**Sublibrary 133**	26.9 ± 1.0	–
Sublibrary 62	25.6 ± 3.3	–	**Sublibrary 136**	18.5 ± 3.2	61.5 ± 2.8
Sublibrary 63	42.3 ± 2.1	–	**Sublibrary 137**	9.1 ± 2.0	50.6 ± 5.6
Sublibrary 64	30.9 ± 4.4	–	**Sublibrary 138**	14.4 ± 3.9	70.5 ± 3.6
Sublibrary 65	16.3 ± 0.9	84.7 ± 5.7	***Sublibrary 140***	1.9 ± 2.0	*34.0 ± 3.5*
Sublibrary 69	24.3 ± 4.6	71.8 ± 5.0			

Active α-amino acids or sublibraries on the 1.0 μM concentration level are highlighted in italics.[a] mean ± SD, n = 3

and 140 were qualified as active (reduction of specific marker binding to ≤ 50%). These four hits, i.e. **SC121–d**, **SC121–f**, **SC121–n** and **SC140–a** [(*S*)-enantiomer of **SC121–d**], are compiled in Table 2 and their structures are displayed in Figure 2. With hit **SC121–n** being distinctly more potent according to deconvolution experiments (reduction of specific marker binding to 22.4 ± 2.3%) as compared to the other three hit compounds **SC121–d** (reduction of specific marker binding to 42.1 ± 3.1%), **SC121–f** (reduction of specific marker binding to 49.8 ± 6.5%) and **SC140–a** (reduction of specific marker binding to 38.9 ± 1.0%) we decided to characterize the pK_i values only for the former compound, **SC121–n**, in full-scale competition experiments together with that of α-amino acids **48** found in the single compound screening. The respective experiments are described in the next section. The entire results of the single compounds screening as well as of the deconvolution experiments of sublibraries 73, 116, 120, 121 and 140 are depicted in Table S1 and S2 of the Supporting Information.

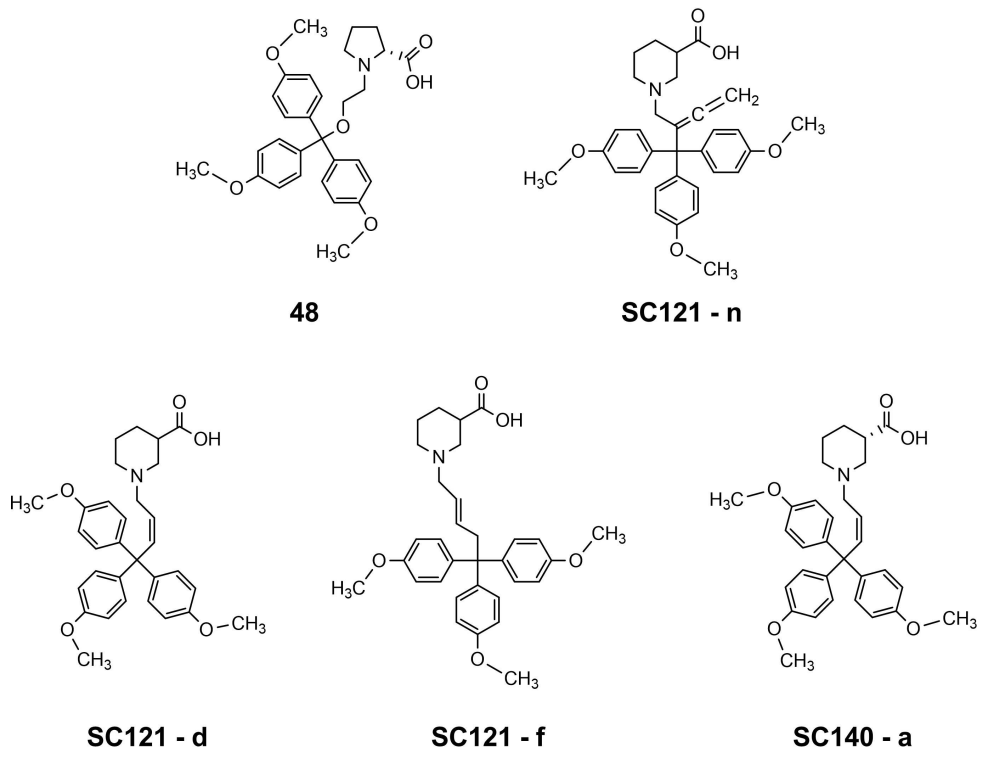

Figure 2. Hit compounds of the GlyT1 MS Binding Assay screening

Affinity characterization of hit compounds in competitive GlyT1 MS Binding Assays

For the determination of the IC_{50} value of α-amino acid **48** and compound **SC121–n** three independent competition experiments per compound were performed according to the recently described GlyT1 MS Binding Assay.[32] Representative inhibition curves are depicted in Figure 3 and the obtained pK_i values calculated from the IC_{50} values are listed in Table 3.

α-Amino acid **48** and **SC121–n** had been developed as part of a study aiming at new inhibitors of the various subtypes of GABA transporter (GAT; see Table 3).[34,35] Comparing the results found in this study regarding the affinity for GlyT1 with the results from literature for GAT inhibition it can be assumed, that compound **48** and **SC121–n** have a higher potential as glycine uptake inhibitors at GlyT1 than as GABA uptake inhibitors provided that the inhibitory potencies at GlyT1 are similar to the binding affinities found for this transporter. Furthermore, **SC121–n** represents a chemical scaffold, which has not been described for GlyT1 inhibitors before and might thus be of interest for the development of new GlyT1 inhibitors. *N*-Substituted prolines have already widely and successfully been explored as GlyT1 inhibitors,[36] however, a combination with a trityl moiety as in **48** has not been reported so far.

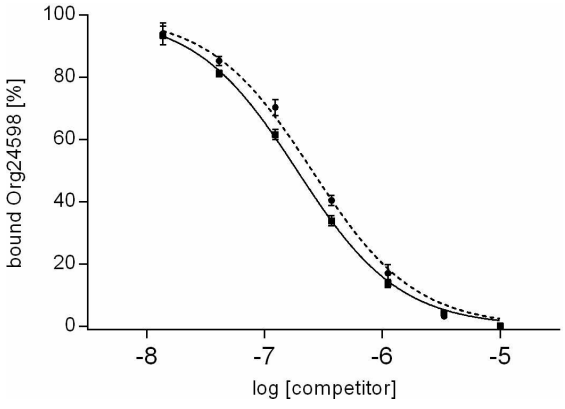

Figure 3. Representative inhibition curves of α-amino acid **48** (▪, solid line) and compound **SC121–n** (●, dashed line). Data represent remaining specific binding of Org24598 to GlyT1 in the presence of increasing concentrations of competitor and are given as mean ± SD of triplicates. Resulting IC_{50} of three independent experiments for each hit compound are transformed into the respective pK_i values for which the mean ± SEM is reported (results are reported in Table 3).

Compound library screening for GlyT2

For the screening of the whole set of test compounds for GlyT2 inhibitors the same procedures as for the screening for the GlyT1 inhibitors was followed, except that this time the GlyT2 MS Binding Assay was employed. Upon screening of the test compounds at a concentration of 10 μM none of the α-amino acids was found to be active but 16 out of the 142 sublibraries reduced marker binding to GlyT2 to ≤ 50% (see Table 4). These 16 sublibraries, which had been identified as active, were

Table 2. Results of the deconvolution experiment of the five most active sublibraries of the 1.0 μM GlyT1 MS Binding Assay screening.

Sublibrary	Sublibrary Constituent	Remaining specific binding of Org24598 at **1.0 μM** [%][a]	Sublibrary	Sublibrary Constituent	Remaining specific binding of Org24598 at **1.0 μM** [%][a]
Sublibrary 73	SC73–a	96.7 ± 3.5	**Sublibrary 121**	SC121–a	100.5 ± 5.0
	SC73–b	104.1 ± 2.3		SC121–b	94.1 ± 10.4
	SC73–c	85.3 ± 10.7		SC121–c	77.2 ± 3.6
	SC73–d	83.8 ± 9.2		*SC121–d*	*42.1 ± 3.1*
	SC73–e	101.5 ± 4.7		SC121–e	71.4 ± 1.4
	SC73–f	105.4 ± 7.7		*SC121–f*	*49.8 ± 6.5*
	SC73–g	107.7 ± 2.5		SC121–g	93.5 ± 5.9
	SC73–h	101.5 ± 4.5		SC121–h	100.2 ± 0.0
	SC73–i	76.0 ± 5.1		SC121–i	92.0 ± 14.1
	SC73–j	105.1 ± 3.4		SC121–j	78.0 ± 3.5
	SC73–k	99.2 ± 3.9		SC121–k	85.7 ± 6.3
	SC73–l	96.4 ± 7.4		SC121–l	104.4 ± 6.0
	SC73–m	84.0 ± 0.5		SC121–m	98.4 ± 4.7
	SC73–n	84.8 ± 7.3		*SC121–n*	*22.4 ± 2.3*
	SC73–o	78.9 ± 2.8		SC121–o	104.4 ± 5.1
	SC73–p	82.7 ± 2.7		SC121–p	103.2 ± 10.0
Sublibrary 116	SC116–a	79.4 ± 4.4	**Sublibrary 140**	*SC140–a*	*38.9 ± 1.0*
	SC116–b	85.1 ± 3.4		SC140–b	89.9 ± 5.5
	SC116–c	73.5 ± 2.1		SC140–c	78.4 ± 3.4
	SC116–d	57.0 ± 2.5		SC140–d	98.0 ± 3.2
	SC116–e	89.9 ± 7.6		SC140–e	85.7 ± 3.6
	SC116–f	91.8 ± 5.1		SC140–f	98.0 ± 5.5
	SC116–g	81.7 ± 5.2		SC140–g	82.5 ± 4.8
	SC116–h	86.4 ± 8.8		SC140–h	104.1 ± 5.5
	SC116–i	78.1 ± 3.1		SC140–i	100.7 ± 5.0
	SC116–j	88.6 ± 7.3		SC140–j	66.9 ± 2.8
	SC116–k	80.9 ± 10.8		SC140–k	100.4 ± 6.1
	SC116–l	86.1 ± 2.9		SC140–l	92.6 ± 12.8
	SC116–m	97.7 ± 6.6		SC140–m	91.1 ± 8.7
	SC116–n	91.5 ± 3.9		SC140–n	81.6 ± 5.1
	SC116–o	90.5 ± 9.9		SC140–o	90.8 ± 3.7
	SC116–p	85.3 ± 2.0		SC140–p	92.0 ± 3.2
Sublibrary 120	SC120–a	96.1 ± 11.8			
	SC120–b	90.2 ± 16.3			
	SC120–c	81.2 ± 1.4			
	SC120–d	89.4 ± 5.5			
	SC120–e	91.5 ± 9.1			
	SC120–f	89.7 ± 7.9			
	SC120–g	78.9 ± 9.4			
	SC120–h	85.8 ± 6.3			
	SC120–i	92.8 ± 2.7			
	SC120–j	86.6 ± 4.7			
	SC120–k	59.5 ± 11.6			
	SC120–l	87.9 ± 7.2			
	SC120–m	89.7 ± 7.9			
	SC120–n	96.9 ± 0.9			
	SC120–o	88.9 ± 3.6			
	SC120–p	92.0 ± 2.8			

Active sublibrary constituents are highlighted in italics. [a] mean ± SD, n = 3

Table 3. pK_i values of hit compounds for GlyT1 found in GlyT1 MS Binding Assay in comparison to potencies/affinities at GABA transporter subtypes.

Compound	pK_i [a] GlyT1	pK_i [a] mGAT1	pIC_{50} [b] hGAT1/mGAT1	hBGT1/mGAT2	hGAT2/mGAT3	hGAT3/mGAT4
48[34]	7.00 ± 0.01	–	3.84*	–	–	4.73*
SC121–n[35]	6.85 ± 0.02	57 %[c]	69 %[d]	77 %[d]	4.37	4.31

GlyT1 pK_i values are given as mean ± SEM (n = 3), all other values are presented as published in the given reference unless otherwise stated. Underlined values represent results, which were measured at murine forms of the GABA transporters. * Values were transformed from IC_{50} to pIC_{50} values. [a] Results determined in binding experiments. [b] Results determined in uptake experiments. [c] Percentages represent remaining specific reporter ligand (NO711) binding in presence of 100 μM test compound in a GAT1 MS Binding Assay.[37] [d] Percentages represent remaining [^{3}H]GABA uptake in presence of 100 μM test compound.

subjected to a second screening process, but under more stringent conditions with a test compound concentration of 1.0 μM (of the constituents of the sublibrary). In this experiment just one of the studied sublibraries, sublibrary 70, was found to

Table 4. Results of the 10 μM and 1.0 μM GlyT2 MS Binding Assay screening for the sublibraries fulfilling the selection criteria.

Sublibrary	Remaining specific binding of Org25543 at **10 μM** [%][a]	Remaining specific binding of Org25543 at **1.0 μM** [%][a]	Sublibrary	Remaining specific binding of Org25543 at **10 μM** [%][a]	Remaining specific binding of Org25543 at **1.0 μM** [%][a]
Sublibrary 5	29.4 ± 0.3	71.4 ± 7.3	**Sublibrary 88**	43.9 ± 2.0	96.0 ± 3.3
Sublibrary 18	49.6 ± 3.1	78.4 ± 6.8	**Sublibrary 95**	33.2 ± 1.9	90.3 ± 3.8
Sublibrary 69	19.9 ± 5.0	91.4 ± 2.5	**Sublibrary 96**	29.0 ± 2.2	86.9 ± 5.3
Sublibrary 70	13.0 ± 2.6	*50.0 ± 9.6*	**Sublibrary 104**	47.5 ± 0.5	88.6 ± 2.1
Sublibrary 71	20.5 ± 3.8	*58.7 ± 7.0*	**Sublibrary 106**	41.2 ± 1.1	89.9 ± 0.8
Sublibrary 73	17.2 ± 1.7	87.4 ± 3.6	***Sublibrary 107***	7.3 ± 1.9	*52.9 ± 3.4*
Sublibrary 76	24.3 ± 3.3	95.9 ± 5.6	**Sublibrary 122**	39.8 ± 3.5	79.9 ± 9.9
Sublibrary 77	28.0 ± 2.7	90.6 ± 5.0	**Sublibrary 131**	36.3 ± 3.1	93.5 ± 2.1

Sublibraries active on the 1.0 μM concentration level are highlighted in italics. [a] mean ± SD, n = 3

reduce specific binding of the GlyT2 marker Org25543 to 50% and non below. In addition, two sublibraries (sublibraries 71 and 107) showed values for remaining specific marker binding that were not much above 50% but distinctly lower than those of all others (see Table 4). Hence in addition to sublibrary 70 that had just met the selection criterion (≤50% reduction of specific marker binding) these two sublibraries were subjected to deconvolution experiments. Since these sublibraries had shown only moderate activities at 1.0 μM, we decided to perform the deconvolution experiments at 10 μM. These experiments revealed **SC70–o** (48.9 ± 5.5%), **SC70–p** (10.4 ± 0.4%), **SC71–a** (15.1 ± 1.8%), **SC71–f** (20.7 ± 1.9%), **SC71–m** (44.5 ± 8.7%) and **SC107–f** (23.8 ± 1.3%) to reduce specific marker binding to ≤50% at the studied concentration of 10 μM and were thus considered as hit compounds for GlyT2 (see Table 5 for the screening results). The structures of these hit compounds are given in Figure 4. The characterization of their binding affinities in full-scale competitive GlyT2 MS Binding Assays is described in the next section. The structures of all constituents of the three sublibraries subjected to deconvolution experiments are depicted in Supporting Information Table S3.

Table 5. Results of the deconvolution experiment of the three most active sublibraries of the 1.0 μM GlyT2 MS Binding Assay screening.

Sublibrary	Sublibrary Constituent	Remaining specific binding of Org25543 at **10 μM** [%][a]	Sublibrary	Sublibrary Constituent	Remaining specific binding of Org25543 at **10 μM** [%][a]
Sublibrary 70	SC70–a	91.4 ± 4.3	**Sublibrary 107**	SC107–a	77.7 ± 1.4
	SC70–b	88.9 ± 2.1		SC107–b	83.4 ± 4.5
	SC70–c	85.7 ± 4.6		SC107–c	72.7 ± 14.9
	SC70–d	83.8 ± 1.8		SC107–d	94.2 ± 3.7
	SC70–e	92.3 ± 6.4		SC107–e	94.3 ± 5.3
	SC70–f	69.6 ± 6.1		*SC107–f*	*23.8 ± 1.3*
	SC70–g	63.8 ± 4.9		SC107–g	92.2 ± 3.5
	SC70–h	89.5 ± 8.2		SC107–h	98.9 ± 3.0
	SC70–i	109.8 ± 5.9		SC107–i	96.3 ± 4.2
	SC70–j	78.9 ± 7.4		SC107–j	93.5 ± 1.8
	SC70–k	89.7 ± 2.8		SC107–k	94.9 ± 3.5
	SC70–l	96.9 ± 4.1		SC107–l	105.1 ± 6.8
	SC70–m	64.7 ± 1.4		SC107–m	104.0 ± 6.5
	SC70–n	95.5 ± 3.8		SC107–n	71.2 ± 2.1
	SC70–o	*48.9 ± 5.5*		SC107–o	99.1 ± 6.6
	SC70–p	*10.4 ± 0.4*		SC107–p	111.5 ± 2.0
Sublibrary 71	*SC71–a*	*15.1 ± 1.8*			
	SC71–b	81.7 ± 9.0			
	SC71–c	109.3 ± 10.4			
	SC71–d	93.3 ± 7.7			
	SC71–e	87.3 ± 4.6			
	SC71–f	*20.7 ± 1.9*			
	SC71–g	66.8 ± 10.9			
	SC71–h	96.2 ± 5.5			
	SC71–i	88.2 ± 5.2			
	SC71–j	100.1 ± 4.7			
	SC71–k	112.4 ± 1.6			
	SC71–l	99.1 ± 6.0			
	SC71–m	*44.5 ± 8.7*			
	SC71–n	100.0 ± 3.3			
	SC71–o	81.6 ± 1.6			
	SC71–p	157.7 ± 2.2			

Active sublibrary constituents are highlighted in italics. [a] mean ± SD, n = 3

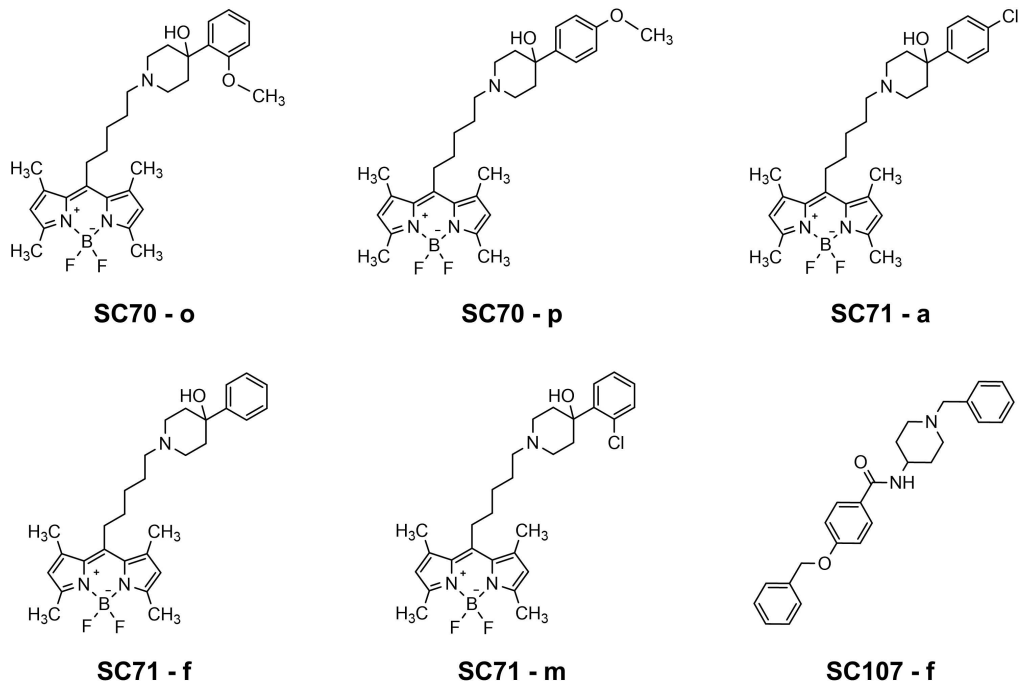

Figure 4. Hit compounds of the GlyT2 MS Binding Assay screening

Affinity characterization of hit compounds in competitive GlyT2 MS Binding Assays

The binding affinities (pK_i) of the six hit compounds **SC70**–**o**, **SC70**–**p**, **SC71**–**a**, **SC71**–**f**, **SC71**–**m** and **SC107**–**f** were determined in competitive GlyT2 MS Binding Assays, which were performed as described recently.[33] Of each hit compound one representative inhibition curve out of three independent competition experiments is depicted in Figure 5 and the obtained pK_i values are listed in Table 6.

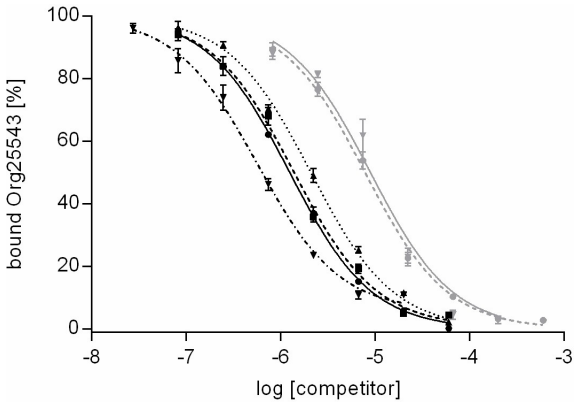

Figure 5. Representative inhibition curves of compounds **SC70–o** (▼, solid line), **SC70–p** (●, solid line), **SC71–a** (▪, dashed line), **SC71–f** (▲, dotted line), **SC71–m** (●, dashed line) and **SC107–f** (▼, dash-dotted line). Data represent remaining specific binding of Org25543 to GlyT2 in the presence of increasing concentrations of competitor and are given as mean ± SD of triplicates. Resulting IC_{50} of three independent experiments for each hit compound are transformed into the respective pK_i values for which the mean ± SEM is reported (results are reported in Table 6).

Compounds **SC70**–**o**, **SC70**–**p**, **SC71**–**a**, **SC71**–**f** and **SC71**–**m** had been primarily developed as fluorescent ligands of the GABA transporters mGAT1-mGAT4.[38] The 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) moiety and its derivatives, an example of which is present in the aforementioned ligands of GlyT2, are known to exhibit excellent fluorescent properties.[40,41] This makes compounds comprising such a fluorescent label valuable tool compounds for various kinds of fluorescence-based bioassays, e.g. fluorescence binding assays[42,43] or fluorescence resonance energy transfer (FRET) experiments.[44,45] Some of the identified GlyT2 ligands comprising a BODIPY subunit might be suitable for such purposes, as well. In GABA uptake experiments the here listed GlyT2 hit compounds exhibited the highest inhibitory potencies (pIC_{50}) at the GABA transporters mGAT2 and mGAT4 for which pIC_{50} values up to 5.35 were found and to some extent also at mGAT3 (see Table 6). In comparison to the GlyT2 affinities (pK_i) it seems that at least **SC70**–**p**, **SC71**–**a** and **SC71**–**f** show higher potential as GlyT2 than as GAT inhibitors. However, one has to keep in mind, that the data for GlyT2 describe binding affinities (pK_i) and not inhibitory potencies (pIC_{50}) at their target, which might differ quite substantially. Further, it is worth to look at the binding affinities of these hit compounds at mGAT1. In comparison to the pK_i values at GlyT2 it can be noticed, that only the affinity of compound **SC71**–**m** is in the same range for GlyT2 and mGAT1 (5.46 ± 0.03 vs. 5.27 ± 0.05). For the other hit compounds, the affinity at GlyT2 is significantly higher than that at mGAT1. Interestingly, for the five GlyT2 hit compounds comprising a BODIPY subunit a general connection between

Table 6. pK_i values of hit compounds for GlyT2 found in GlyT2 MS Binding Assay in comparison to potencies/affinities at GABA transporters.

Compound	pK_i [a] GlyT2	pK_i [a] mGAT1	pIC_{50} [b] mGAT1	mGAT2	mGAT3	mGAT4
SC70–o[38]	5.42 ± 0.01	4.25	4.91	5.11 ± 0.07	5.12 ± 0.06	5.20 ± 0.06
SC70–p[38]	6.40 ± 0.07	63 %[c]	4.63	5.11 ± 0.05	4.94	5.15 ± 0.05
SC71–a[38]	6.18 ± 0.05	56 %[c]	4.96	5.30 ± 0.07	4.82	4.85
SC71–f[38]	5.94 ± 0.08	4.57	4.84	5.07 ± 0.09	4.87	5.09 ± 0.07
SC71–m[38]	5.46 ± 0.02	5.27 ± 0.05	4.90	5.35 ± 0.04	5.12 ± 0.08	5.25 ± 0.03
SC107–f[39]	6.67 ± 0.03	–	–	–	–	–

GlyT2 pK_i values are given as mean ± SEM (n = 3), all other values are presented as published in the given reference. [a] Results determined in binding experiments. [b] Results determined in uptake experiments. [c] Percentages represent remaining specific reporter ligand (NO711) binding in presence of 100 μM test compound in a GAT1 MS Binding Assay.[37]

structure and activity appears to become evident. The binding affinity of the parent compound **SC71–f** devoid of any substituent at the phenyl ring in 4-position of the piperidine moiety is characterized with a pK_i value of 5.94 ± 0.08. This binding affinity increases, when a substituent at said phenyl ring is present in the para (Cl, **SC71–a**, pK_i = 6.18 ± 0.05; OCH_3, **SC70–p**, pK_i = 6.40 ± 0.07) but decreases when it is located at the ortho position (Cl, **SC71–m**, pK_i = 5.46 ± 0.02; OCH_3, **SC70–o**, pK_i = 5.42 ± 0.01).

Compound **SC107–f** represents the most affine compound we found in our screening for GlyT2 binders, its pK_i amounting to 6.67 ± 0.03. With the reporter ligand Org25543 (Figure 1), it has a 4-(benzyloxy)benzamide in common and has already been reported before alike related benzamides as GlyT2 inhibitor.[39,46] In a glycine uptake screening for GlyT2 it showed a reduction/inhibition of [^{3}H]glycine uptake of 74 % (≙ 26 % remaining [^{3}H]glycine uptake) at a concentration of 1.0 μM which seems to be well in agreement with the binding affinity determined in this study.[39]

Conclusion

A compound library screening comprising 2439 substances has been performed to identify new inhibitors for the neurotransmitter transporters GlyT1 and GlyT2. GlyT1 and GlyT2 MS Binding Assays recently reported by us were employed to study either individual constituents of the compound library (α-amino acids) or, even more important, sublibraries containing 16 compounds. Thereby the screening procedure appeared to be very efficient and highly reliable in identifying hit compounds.

For GlyT1 we were able to identify the α-amino acid **48** as well as compounds **SC121–d, SC121–f**, **SC121–n** and **SC140–a** as hit compounds (see Figure 2). Compounds **SC121–d, SC121–f** and **SC140–a** were able to reduce specific marker binding to 42.1 ± 3.1 %, 49.8 ± 6.5 % and 38.9 ± 1.0 %, respectively, at a test concentration of 1.0 μM. For the two best inhibitors we identified for GlyT1, **48** and **SC121–n**, pK_i values of 7.00 ± 0.01 and 6.85 ± 0.02 were found, respectively. In comparison to already known GlyT1 inhibitors like Org24598 (K_d = 16.8 ± 2.2 nM[32]) or ALX5407 (pK_i = 8.89 ± 0.04[32]) the affinity is lower of about one to two orders of magnitude. Still, the here described substances possess new structural scaffolds (especially **SC121–n**) for GlyT1 binders and could thus be of interest as a starting point for the development of more potent GlyT1 inhibitors.

The screening for GlyT2 binders yielded the six hit compounds **SC70–o**, **SC70–p**, **SC71–a**, **SC71–f**, **SC71–m** and **SC107–f** (see Figure 4). Compounds **SC70–o**, **SC70–p**, **SC71–a**, **SC71–f** and **SC71–m** displaying pK_i values of 5.42 ± 0.01, 6.40 ± 0.07, 6.18 ± 0.05, 5.94 ± 0.08 and 5.46 ± 0.02, respectively, are all possessing a difluoroboraindacene subunit known for excellent fluorescent properties. Hence, these compounds could serve as tool compounds for fluorescence-based experiments at GlyT2 to further investigate the inhibition of GlyT2 and pharmacological consequences thereof, as for example internalization and recycling processes. Alike the reporter ligand Org25543 compound **SC107–f** belongs to a known class of GlyT2 inhibitors, which is based on central benzamide subunit. With a pK_i value of 6.67 ± 0.03 it exhibits the highest affinity for GlyT2 among the identified hit compounds. Though, in former studies this compound has already been investigated for its inhibitory potency at GlyT2,[39,46] this is the first time that its binding affinity has been determined. Thereby, the results of the functional assay match reasonably well the here described binding affinity. Furthermore, the identification of this known GlyT2 inhibitor (**SC107–f**) as hit compound clearly underlines the reliability and effectivity of the here performed GlyT2 MS Binding Assay screening.

Overall, it can be concluded that the aim of this study to identify new inhibitors for GlyT1 and GlyT2 which exhibit affinities that are at least in the low micromolar to submicromolar range was fully achieved. Additionally, it could be demonstrated that MS Binding Assays, as in the present case for GlyT1 and GlyT2, are a powerful tool for the examination of compound libraries of reasonable size in a straightforward manner.

Experimental Section

Chemicals

Org24598 (*N*-methyl-*N*-[(3*R*)-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propyl]glycine) as lithium salt (purity ≥ 98 %, HPLC), ALX5407 (*N*-[(3*R*)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl) propyl]-*N*-methylglycine) as hydrochloride (purity ≥ 98 %, HPLC)

and Org25543 (*N*-[[1-(dimethylamino)cyclopentyl]methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide) as hydrochloride (purity ≥99%, HPLC), were purchased from Tocris (Bristol, UK). [$^2H_5$]Org24598[32] and [$^2H_7$]Org25543[33] were synthesized in-house. All other test compounds were either synthesized in-house or were acquired by purchase from common supplier. Water was exclusively obtained from a Sartorius arium pro ultrapure water system (Sartorius, Göttingen, Germany). HPLC grade methanol from VWR Prolabo (Darmstadt, Germany) was used for washing the glass fiber filters in GlyT1 and GlyT2 MS Binding Assays, whereas LC-MS grade methanol was used for elution of marker from target-marker-complexes in the GlyT1 MS Binding Assay only. For elution of marker in the GlyT2 MS Binding Assays and for the mobile phase in chromatography for both assays LC-MS grade acetonitrile from VWR Prolabo (Darmstadt, Germany) was used. All other chemicals like buffer salts were purchased in analytical grade.

LC-ESI-MS/MS

Quantification by LC-ESI-MS/MS was performed using an API5000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany). Detailed LC conditions and MS instrument settings are exactly as described previously for GlyT1 and GlyT2 MS Binding Assays.[32,33]

GlyT1 and GlyT2 membrane preparations

Membrane preparations of CHO-K1 cells stably expressing hGlyT1c and HEK 293 cells stably expressing hGlyT2 were prepared and applied as recently described.[32,33]

General procedure of compound and library screening

A total of 2439 compounds were available for screening which were either tested as single compounds (α-amino acid) or as sublibraries each with 16 compounds (all other compounds) in competitive binding assays. For the following steps 10-fold concentrated solutions in incubation buffer of the single compounds or sublibraries of the applied concentrations were prepared to use for the experiments. In a first screening experiment the α-amino acids or sublibraries were tested at a concentration of 10 μM (concentration in working solution: 100 μM; in sublibraries the concentration of every constituent accounted for 100 μM in the working solution). Those which reduced specific reporter ligand binding to ≤50% (for GlyT1 and GlyT2) proceeded to a second screening round in which they were tested at a concentration of 1.0 μM (concentration in working solution: 10 μM; in sublibraries the concentration of every constituent accounted for 10 μM in the working solution). The single compounds or sublibraries, which reduced the specific marker binding to ≤50% (for GlyT1 and GlyT2), in this second screening process, were identified as active entities. Unless no active entity was identified in this second screening process further studies had to focus on the active entities found in the first screening round. Single α-amino acids, which fulfill the selection criteria, were directly characterized for their IC_{50} values in full scale competition experiments and therefrom the pK_i were determined. If a sublibrary was identified as active, a deconvolution experiment was performed of its single constituents at the same test compound concentration. The pK_i values were determined of those constituents, which fulfilled the selection criterion, i.e., reduction of specific marker binding to ≤50% at the respective test compound concentration.

GlyT1 competitive MS Binding Assays – Screening experiments

The screening experiments were basically performed as described previously.[32] GlyT1 membrane preparations were incubated with Org24598 (15 nM) and test compound or sublibrary (10 μM and 1.0 μM) in incubation buffer (10 mM HEPES, 120 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, pH 7.5) in polypropylene 96 well plates (1.2 mL well volume, Sarstedt, Nümbrecht, Germany) in a total volume of 250 μL at 22 °C in a Stuart® Microtitre Plate Shaker Incubator SI505 (Bibby Scientific Limited, Staffordshire, Great Britain) for 1 h. Each test compound or sublibrary was tested in triplicates. Additionally, control samples were prepared in triplicates to define total binding of Org24598 in absence of any competitor and non-specific binding was defined as remaining binding in presence of 20 μM ALX5407. Incubation was terminated by vacuum filtration (Multi Well Plate Vacuum Manifold, Pall, Dreieich, Germany) over 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 μm, 350 μL; Pall Corporation, Port Washington, New York, US) which were, before their use, washed with 3×200 μL water, 3×200 μL methanol, preincubated with 200 μL per well of an 1% (*m*/*m*) aqueous Tween20 solution at room temperature for 2 h and, finally exempted from the Tween20 solution by vacuum filtration. After transferring 210 μL aliquots of the binding samples onto the filters, the filters with the target-marker-complexes on it were washed with ice-cold 154 mM ammonium acetate buffer (3×200 μL; pH 7.4) and subsequently, dried at 50 °C for 1 h. After drying the target bound reporter ligand is liberated from the protein by applying methanol containing 100 pM [$^2H_5$]Org24598 as internal standard to the filters (3×70 μL) and collecting the eluate after vacuum application in another 96 well plate. The methanolic eluates were evaporated at 50 °C under N_2 flow by means of a MiniVap Microevaporator (Porvair Sciences Limited, Norfolk, UK) and the remaining residues were reconstituted with 210 μL mobile phase (5.0 mM ammonium bicarbonate buffer, pH 7.8/acetonitrile (55:45, v/v)). Finally, the resulting samples were subjected to the LC-ESI-MS/MS for quantification of bound reporter ligand at GlyT1.

GlyT2 competitive MS Binding Assays – Screening experiments

The screening experiments were basically performed as described previously.[33] GlyT2 membrane preparations were incubated with Org25543 (10 nM) and test compound or sublibrary (10 μM and 1.0 μM) in incubation buffer (10 mM HEPES, 120 mM NaCl, 2.0 mM KCl, 1.0 mM MgC_{l2}, 1.0 mM $CaCl_2$, pH 7.5) in polypropylene 96 well plates (1.2 mL well volume, Sarstedt, Nümbrecht, Germany) in a total volume of 250 μL at 37 °C in a Julabo SW-20 C water bath (Julabo GmbH, Seelbach, Germany) for 1 h. Each test compound or sublibrary was tested in triplicates. Additionally, control samples were prepared in triplicates to define total binding of Org25543 in absence of any competitor and non-specific binding was defined as remaining binding in presence of GlyT2 membrane preparations, which were preincubated at 60 °C for 1 h in a water bath ("Heat-Shock"). Incubation was terminated by vacuum filtration (Multi Well Plate Vacuum Manifold, Pall, Dreieich, Germany) over 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 μm, 350 μL; Pall Corporation, Port Washington, New York, US) which were, before their use, washed with 3×200 μL water, 3×200 μL methanol, preincubated with 200 μL per well of an 0.5% (*m*/*m*) aqueous polyethylenimine solution (PEI) at room temperature for 2 h and, finally exempted from the PEI solution by vacuum filtration. After transferring 210 μL aliquots of the binding samples onto the filters, the filters with the target-marker-complexes on it were washed with ice-cold 154 mM ammonium acetate buffer (4×150 μL; pH 7.4) and subsequently, dried at 50 °C for 1 h. After drying the target

bound reporter ligand is liberated from the protein by applying acetonitrile containing 125 pM [$^2H_7$]Org25543 as internal standard and 0.2% dimethylacetamide (DMA) to the filters (3×70 µL) and collecting the eluate after vacuum application in another 96 well plate. Additionally, 5.0 mM ammonium bicarbonate buffer is added to the collected eluates (52.5 µL per well) to obtain a sample solvent matching the mobile phase (ammonium bicarbonate buffer (pH 7.8) and acetonitrile 20:80, *v/v*). Finally, the resulting samples were subjected to the LC-ESI-MS/MS for quantification of bound reporter ligand at GlyT2.

GlyT1 and GlyT2 competitive MS Binding Assays – Determining binding affinities of hit compounds

Full-scale competitive MS Binding Assays for GlyT1 and GlyT2 were performed as recently published.[32,33] Instead of using a single concentration as described above for the screening experiments the hit compounds were investigated for at least seven concentration levels, which covered approximately three orders of magnitude (in triplicates).

Data analysis

In screening experiments the results of specific marker binding at the different concentration levels of inhibitors are given as the mean ± standard deviation (SD). The results of the competitive MS Binding Assays (pK_i values) for the hit compounds are given as the mean ± standard error of the mean (SEM; at least three experiments). To determine the marker concentration in binding experiments, an individual calibration function was established for every binding experiment as described for the corresponding MS Binding Assays.[32,33] Based on the obtained calibration functions the bound marker concentrations were determined with Analyst v. 1.6.1 Software. The difference between total and non-specific binding was defined as the specific binding of the reporter ligand. Using the *One Site – Fit logIC$_{50}$* non-linear regression tool of Prism v. 6.07 (GraphPad Software, San Diego, CA, USA) the data from the binding experiments were analyzed to obtain sigmoidal competition curves. The top level (total binding in absence of test compound) was set to 100% and the bottom level (non-specific binding) was set to 0%. The hit compound concentration, which cause a reporter ligand inhibition to 50%, was defined as IC_{50} value (half maximal inhibitory concentration). The determined IC_{50} value was then transferred into K_i values (inhibition constant of the test compound) using the Cheng-Prusoff equation $K_i = IC_{50}/(1 + [L]/K_d)$, with [L] = reporter ligand concentration in competition experiment and K_d = dissociation constant of the reporter ligand for the target protein. For GlyT1 competition experiments an Org24598 concentration of 15 nM and a $K_d = 16.8 \pm 2.2$ nM[32] and for GlyT2 competition experiments an Org25543 concentration of 10 nM and a $K_d = 7.45 \pm 0.55$ nM [33] were used. Finally, for the determined K_i values of the hit compounds the corresponding pK_i values were calculated.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: binding assay · library screening · glycine transporter · liquid chromatography · mass spectrometry

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